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Nucleoside Transport in Cardiac Myocytes

by

Timothy Peter Heaton

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF Master of Science

Department of Pharmacology

EDMONTON, ALBERTA

Fall, 1986

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ABSTRACT

Adenosine is an important regulatory compound in the heart. Its actions are mediated via receptors on the extracellular surface of cells and are terminated by its transport into cells and subsequent metabolism. The nucleoside transport system plays a key role in the termination of the actions of adenosine and therefore, drug-induced inhibition of transport potentiates adenosine actions. Dissociated ventricular myocytes from guinea pig and rat were used for the present study of the nucleoside transport system. The binding of the high affinity probe, [^{3}H]nitrobenzylthioinosine ([^{3}H]NBMPR), was compared with measurements of initial rates of nucleoside influx to (a) characterize the transport process, (b) determine if NBMPR may be a useful high affinity probe for studying the nucleoside transport system in cardiac cells and (c) explore species differences in the nucleoside transport system of myocytes.

In guinea pig and rat myocytes, [^{3}H]NBMPR was bound with high affinity to a single class of sites with Kd values (95% confidence limits) of 0.76 (0.58 - 1.00) nM and 0.40 (0.29 - 0.56) nM, respectively and which had maximum binding capacities (± S.E.) of 826,000 ± 90,000 and 437,000 ± 45,000 molecules per cell, respectively. Adenosine and 2-chloroadenosine entered cells by a saturable and inhibitable process. The Km (95% confidence limits) and Vmax (± S.E.) values for adenosine transport into guinea pig

myocytes were 146 (101 - 210) μM and 24.2 ± 1.4 pmol/10⁶cells/s, respectively. The maximum velocity of adenosine transport into rat myocytes (Vmax = 7.1 ± 0.2 pmol/10⁶cells/s), was lower than that with guinea pig, however, adenosine possessed a higher affinity for the transport process in rat cells (Km = 50 (38 - 67) μM), as compared to those in guinea pig. The Km and Vmax values for 2-chloroadenosine transport into guinea pig myocytes were 36 (34 - 39) μM and 11.7 ± 0.1 pmol/10⁶cells/s, respectively.

Dipyridamole was a more potent inhibitor of [³H]NBMPR binding in guinea pig myocytes (Ki = 75 (44 - 132) nM) than in rat myocytes (Ki = 1700 (1100 - 2500) nM). In addition, dipyridamole was a more potent inhibitor of adenosine transport into guinea pig myocytes (Ki = 78 (60 - 101) nM) than into rat myocytes (Ki = 3600 (1600 - 8500) nM). The affinities of other drugs (dilazep, diazepam, adenosine and 2-chloroadenosine) for the transport system were also examined.

In myocytes, the maximum NBMPR site density was predictive of nucleoside transport capacity. The results indicate that NBMPR is a useful high affinity probe for the nucleoside transport system in cardiac cells because drug potencies for the inhibition of NBMPR binding were similar to potencies for the inhibition of nucleoside transport. This study also demonstrated in cardiac cells species heterogeneity of the nucleoside transport system. Guinea pig and rat myocytes are different in transport site density

and transport capacity, as well as differences in drug affinities for the transport system. If these differences could be found in the same species, or the same tissue, transport inhibitors might be designed to modify selectively the actions of adenosine.

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I. INTRODUCTION

Adenosine is an endogenous nucleoside, comprised of the purine base adenine linked to ribose. It is an extremely ubiquitous metabolic intermediate which may be involved in nucleic acid biosynthesis, ATP formation and methylation reactions involving the methyl donor S-adenosylmethionine. The hypotensive, cardiac depressive, sedative and antispasmodic effects of adenosine were first described in 1929 (Drury and Szent-Gyorgi 1929). Recently, adenosine has come to be known for its many regulatory functions in the body (for reviews see Stone 1981; Daly 1982; Stone 1983; Snyder 1985). Some of the regulatory functions attributed to the nucleoside include the autoregulation of organ blood flow, regulation of cardiac muscle excitability, control of neuronal excitability, presynaptic neuromodulation, regulation of postsynaptic receptor sensitivity, central regulation of breathing and possibly, the regulation of sleep and wakefulness. The physiological actions of adenosine are believed to be mediated through a number of extracellular receptors. It crosses cell membranes by membrane-located facilitated diffusion systems that possess a broad specificity for nucleosides. Nucleosides have also been shown to cross brush border membranes in the kidneys and the intestines by sodium-dependent, active transport systems. The removal of adenosine from the vicinity of its extracellular receptors by transport systems, and subsequent intracellular metabolism, is implicated as its principal

mechanism of clearance. Agents that block the transport of adenosine into cells, and thus prevent its inactivation, would be expected to potentiate the effects of adenosine at its extracellular receptor sites.

This introductory chapter begins with a brief overview of the biological actions of adenosine, followed by a description of adenosine receptor classification. This will be followed by a review of the enzymatic metabolism and release of adenosine in cardiac tissue. The nucleoside transport system and drug-induced inhibition of this system will be described, including a review of the evidence supporting the use of nitrobenzylthioinosine (NBMPR) as a high affinity probe for studying the nucleoside transport system. Evidence will be presented for the existence of nucleoside transport system heterogeneity. Finally, the rationale for investigating the nucleoside transport system in cardiac tissue will be discussed.

A. Actions of Adenosine

Adenosine is recognized as a regulator of many physiological functions in the body. It is known to have profound effects on the cardiovascular system involving both cardiac function and vascular resistance. Adenosine has been shown to (1) depress both the rate and force of contraction of the heart (Urthaler *et al.* 1981; Evans *et al.* 1982), (2) depress atrioventricular nodal conduction (Bellardinelli *et al.* 1980; Dimarco *et al.* 1983;

Bellardinelli *et al*. 1983), and (3) inhibit atrial and ventricular automaticity (Szentmiklosi *et al*. 1980; Rosen *et al*. 1983). Adenosine has been proposed as a diagnostic tool, as well as a treatment, for paroxysmal supraventricular tachycardias that involve the A-V node in the re-entry pathway (Dimarco *et al*. 1983). An advantage to using adenosine is that it is short-lived, and thus, produces limited side-effects. One of the most documented funtions of adenosine concerns its role in the regulation of coronary blood flow (Berne 1963; Berne 1980; Mustafa 1980). Under conditions of myocardial hypoxia, adenosine is released from myocardial cells (Su 1975; Dewitt *et al*. 1983) resulting in coronary vasodilation and consequently, an increase in blood flow to hypoxic regions of the heart.

The adenosine hypothesis for regulation of blood flow may also apply in other organs (Berne *et al*. 1983; Schutz *et al*. 1983). In brain tissue, adenosine is released during periods of ischemia (Winn *et al*. 1979), hypoxia (Winn *et al*. 1981; Hoffman *et al*. 1984) or seizures (Winn *et al*. 1980); these are periods of decreased oxygen supply or increased oxygen demand and represent times when increased cerebral blood flow would be beneficial. Adenosine is involved in the regulation of blood flow to skeletal muscle (Berne *et al*. 1983), adipose tissue (Sollevi and Fredholm 1981), intestine (Granger and Norris 1980), spleen (Schutz *et al*. 1983), and kidneys (Spielman and Thompson 1982; Kassell *et al*. 1983; Osswald 1983; Schutz *et al*. 1983).

Adenosine also exerts actions on nonvascular smooth muscle. It relaxes ileum (Davies *et al*. 1982; Baer *et al*. 1984), as well as trachea and bronchial preparations *in vitro* (Coleman 1976; Farmer and Farrar 1976; Karlsson *et al*. 1982). In contrast, contraction of guinea pig trachea with intrinsic tone (Fredholm 1980; Advenier *et al*. 1982), bronchoconstriction in the anaesthetised rat (Pauwels and Van der Straeten 1983), and contraction of human bronchial smooth muscle *in vitro* (Davis *et al*. 1982; Dahlen *et al*. 1983) have been reported. In human asthmatic subjects, adenosine caused a concentration-related bronchoconstriction, with no significant effect on control subjects (Cushley *et al*. 1983), evidence that implies a mediatory role of the nucleoside in asthma (Church *et al*. 1985). There has also been proposed a modulatory role for adenosine in mediator release from mast cells and basophils (Marquardt *et al*. 1978; Marone *et al*. 1979; Church *et al*. 1985).

Recently, the role of adenosine as a neuromodulator has been considered (Fredholm and Hedqvist 1980; Gustafsson 1980; Phillis and Wu 1983; Stone 1983; Williams 1984; Snyder 1985). ATP has been shown to be released with catecholamines following sympathetic nerve stimulation (Fredholm 1976; Fredholm and Hedqvist 1980). In addition, Morel and Meunier (1981) obtained a simultaneous release of ATP and acetylcholine from synaptosomes from stimulated cholinergic nerves. ATP is believed to be converted to

adenosine by phosphohydrolases which are located extracellularly (Schrader 1983). Adenosine has been shown to modulate neurotransmitter activity both pre- and postsynaptically (Snyder 1985; Stjarne and Astrand 1985).

Presynaptically, adenosine has been found to inhibit both the release of noradrenaline (Clanachan *et al*. 1977; Phillis and Wu 1981; Fredholm *et al*. 1983; Jonzon and Fredholm 1984) and acetylcholine (Ginsborg and Hirst 1972; Phillis and Wu 1981) from nerve terminals. Postsynaptically, adenosine has been shown to regulate the sensitivity of postsynaptic receptors (Sattin and Rall 1970; Daly 1976; Fredholm and Hedqvist 1980).

Adenosine may function as an inhibitory neuromodulator in the CNS (Phillis and Wu 1981; Williams 1984; Snyder 1985). Reports of inhibition of neurotransmitter release by nucleosides in the CNS have been made for Ach, dopamine, noradrenaline, γ-aminobutyric acid (GABA), glutamate and serotonin (Williams 1984). Adenosine and related analogues have been shown to be potent depressants of neuronal firing in rat cerebral cortex (Phillis *et al*. 1979b), guinea pig olfactory cortex (Okada and Kuroda 1980), and rat hippocampus (Siggins and Schubert 1981; Lee and Schubert 1982). In general, they have an inhibitory action on nerve transmission in the CNS (Snyder 1985).

A wide variety of behavioral effects have been reported for adenosine (Yarbrough and McGuffin-Clineschmidt 1981; Barraco 1985). Adenosine and its analogues produce profound

effects on arousal levels (Crawley *et al.* 1981; Dunwiddie and Worth 1982; Barraco 1985) and it has been proposed that the nucleoside plays a role in sleep and wakefulness cycles (Virus *et al.* 1983). In addition to its sedative properties, parental injections of adenosine produce antinocisponsive effects (Crawley *et al* 1981), hypothermia (Baird-Lambert *et al.* 1980; Wager-Srdar *et al.* 1983), hypnogenic activity (Radulovacki *et al.* 1982), muscle relaxation (Baird-Lambert *et al.* 1980) and anticonvulsant action (Dunwiddie and Worth 1982). The adenosine antagonist theophylline produced marked convulsant effects in guinea pigs (Persson and Erjefalt 1981) and nucleosides antagonized seizures produced by caffeine, another methylxanthine (Marangos *et al.* 1981). Much of the behavioral effects of adenosine and related nucleoside analogues have been linked to their inhibitory neuromodulatory role in the CNS (Barraco 1985).

Additional biological effects of adenosine include antilypolysis (Fredholm and Sollevi 1985), increased steroidogenesis in adrenals and Leydig cells (Londos *etal.* 1980) and inhibition of platelet aggregation (Hourani and Cusack 1985). Also it is possible that the build-up of adenosine following cardiac ischemia could have a beneficial effect on blood clotting during this condition (Snyder 1985).

B. Adenosine Receptors

Much work has gone into the characterization and classification of the receptors through which adenosine exerts its actions. Sattin and Rall (1970) were the first to show that exogenously applied adenosine and some adenine nucleotides were potent stimulants of cAMP accumulation in brain slices, and that this effect was inhibited by methylxanthines. They proposed the existence of external receptors for adenosine that mediate stimulation of adenylate cyclase.

Londos and Wolff (1977) first classified adenosine receptors in various tissues and cell types on the basis of adenylate cyclase activity and stereochemical specificity of agonists. They proposed the existence of adenosine receptors of two types: the R-site and the P-site. The extracellular R-site does not tolerate substitutions on the ribose moiety of the adenosine molecule and was believed to mediate an activation of adenylate cyclase. The P-site required an intact purine ring and was believed to be intimately associated with the adenylate cyclase catalytic subunit, indicating accessibility only from the interior of the cell (Van Calker *et al.* 1979; Daly 1982). Methylxanthines, such as theophylline, inhibited the actions of adenosine at the extracellular R-site but not at the intracellular P-site.

Subsequently, two laboratories developed independently an adenosine receptor classification based on the effects

and rank order of potencies of adenosine and related analogues on adenylate cyclase activity (Van Calker *et al*. 1979; Londos *et al*. 1980). Van Calker and his collegues (1979) designated receptors with inhibitory and stimulatory effects, respectively, on adenylate cyclase activity, as A_1 and A_2 receptors. Londos and assosiates (Londos *etal*. 1980) called these sites Ra (causing activation of adenylate cyclase) and Ri (causing inhibition of adenylate cyclase). Working with three adenylate cyclase systems, where adenosine either stimulated cAMP accumulation (liver and Leydig cells) or inhibited cAMP accumulation (adipocytes), Londos and his co-workers (1980) investigated the rank order of potency of adenosine and a number of analogues on adenylate cyclase. The N⁶-substituted analogue, N⁶-phenylisopropyladenosine (PIA) was more potent than adenosine, which was more potent than the amide-substituted 5'-carboxyl analogue, 5'-N-ethylcarboxamideadenosine (NECA), at inhibiting cyclase activity in fat cells. Liver cell enzyme and Leydig cell enzyme showed the reverse order of potencies: NECA > adenosine > PIA. The L-stereoisomer of PIA has been shown to be 30- to 500-fold more potent than the D-stereoisomer for the A_1 receptor, whereas, they display only a 1- to 10-fold potency difference at A_2 receptors (Daly 1982; Paton and Olsson 1985). Both NECA and PIA show only weak activity at the intracellular P-site (Daly 1982; Londos *et al*. 1980).

A shared characteristic of A_1 and A_2 receptors is their susceptibility to inhibition by methylxanthines. However, the antagonists developed to date show no specificity for these two types of receptors. The rank order of potencies of some xanthines for both A_1 and A_2 adenosine receptor-mediated effects on adenylate cyclase are: 8-phenyltheophylline > 1'3-dibutylxanthine > 3-isobutyl-1-methylxanthine > theophylline > caffeine > theobromine (Daly 1982). The xanthines do not affect P-site inhibition of adenylate cyclase activity (Londos *et al.* 1980; Daly 1982).

There are a number of problems associated with the present adenosine receptor classification. Historically, these have been based on agonist binding potencies and their order of potency either to stimulate or inhibit cAMP production (Collis 1985). However, it is unlikely that all of the actions of these compounds are mediated through interactions with adenylate cyclase (McKenzie *et al.* 1977; Fredholm 1982). In some smooth muscle preparations (for example, longitudinal muscle of the rabbit intestine), there is evidence that the smooth muscle relaxant action of adenosine is not mediated by inhibition of adenylate cyclase or changes in cAMP levels (McKenzie *et al.* 1977; Baer *et al.* 1983). In addition, antagonists have not yet been developed which show selectivity for either receptor, under the present classification. Based on these observations, some researchers believe that the present receptor subdivision

must be treated with caution (Collis 1985).

A working hypothesis for adenosine receptor classification was proposed (Stone 1985) based upon adenosine analogue affinity and potency, with no implication for the involvement of adenylate cyclase. A_1 receptors possess a higher sensitivity to N^6-substituted purines, such as PIA, than to 5'-carboxamide analogues, such as NECA. The relative potency of L-PIA to D-PIA at A_1 sites should be no less than 10-fold. A_2 receptors were defined as those that possess a higher sensitivity to carboxamides than N^6-substituted analogues, and where there is less than 10-fold stereoselectivity for isomers of PIA. This thesis will continue with the A_1/A_2 convention as outlined by Stone (1985).

Adenosine receptors have been identified in coronary smooth muscle, atrial and ventricular cardiac muscle and on sympathetic nerve terminals. Relative potencies of agonists demonstrate that the vasodilatory action of adenosine on coronary smooth muscle is mediated through a receptor resembling the A_2 subtype (Collis and Brown 1983; Kusachi *et al*. 1983). The rank order of potency for adenosine and some of its analogues was found to be NECA > cyclopropyl adenosine-5'-uronamide (NCPCA) >> 2-chloroadenosine > L-PIA > CHA > adenosine > D-PIA. Collis and Brown (1983) showed no correllation between coronary vasodilation and adenylate cyclase activity, while other workers showed a stimulation of cAMP accumulation (Kusachi *et al*. 1983). Adenosine

receptors in atrial heart tissue have been found to resemble the A_1 subtype (Collis 1983), since the potency of L-PIA was some 100-fold greater than that of D-PIA. Studies of adenosine receptor binding in ventricular tissue have been undertaken with adenosine analogues (Linden 1984; Linden *et al.* 1985), and these sites appear to resemble the A_1 adenosine receptor. In addition, presynaptic adenosine receptors associated with reduction of noradrenaline release from stimulated sympathetic nerves have been classified as A_1 receptors (Brown and Collis 1983), based on the following agonist potencies: L-PIA >= NECA = 2-chloroadenosine > D-PIA (L-PIA was 60-fold more potent than D-PIA). It is clear that much work is needed, including the development of selective adenosine receptor antagonists, in order to classify further adenosine receptors in heart. The functional interaction of adenosine receptors in the heart and the nucleoside transport system will be discussed later in this thesis.

C. Metabolism of Adenosine in Heart

As will become clear later in this thesis, the metabolism of adenosine influences greatly the nucleoside transporter-mediated accumulation of this nucleoside in cells. The regulation of the synthesis and catabolism of adenosine in the heart is very complex. A number of enzymatic pathways in cardiac myocytes, smooth muscle and endothelial cells are responsible for the maintenance of

adenosine concentrations according to the metabolic requirements of the organ (Daly 1982; Schrader 1983).

There are two major pathways through which adenosine is formed: the adenosine phosphate pathway or the methylation pathway (Daly 1982; Schrader 1983). The relative significance of each seems to depend upon the metabolic demand of the heart cells. A third pathway for the formation of adenosine is through cAMP; however, the significance of this pathway is unknown (Schrader 1983).

The ATP pathway is responsible for the dephosphorylation of nucleotides to 5'AMP and subsequent dephosphorylation to adenosine by 5'-nucleotidase or alkaline phosphatase (Daly 1982; Schrader 1983). These enzymes are located both intracellularly and extracellularly. However, the major site of adenosine production is intracellular by the endo-enzyme (Schrader 1983). Since the Michaelis constant (Km) for alkaline phosphatase is very high (> 250 μM), it is unlikely that this enzyme activity has any significance under physiological conditions (Arch and Newsholme 1978). 5'-Nucleotidase is strongly inhibited by ATP, ADP, and creatine phosphate (Rubio *et al.* 1979); therefore, this enzyme is normally almost fully inhibited. An increase in heart work would lead to an increase in the utilization of energy (ATP, ADP, creatine phosphate), with a resultant increase in 5'-AMP levels. The increase in substrate and increased activity of 5'-nucleotidase would lead to elevated

adenosine production (Rubio *et al*. 1979; Berne *et al*. 1983; Schrader 1983). Thus, it is evident that adenosine formation via the adenosine phosphate pathway is significant during times of energy deficit.

As stated previously, adenosine formation from 5'-AMP most likely occurs within the myocardial cell. The function of the ecto-nucleotidases seem to be for the dephosphorylation of adenine nucleotides which are released into the extracellular space (Schrader 1983). ATP has been reported to be released from skeletal muscle, neuromuscular junctions, endothelial cells and as co-transmitters at nerve synapses (Burnstock 1983; Schrader 1983; Snyder 1985). Ecto 5'-nucleotidase serves as a rapid means of degrading biologically active adenine nucleotides to adenosine (Schrader 1983), which then may be taken up by cells through the nucleoside transport system (Paterson *et al*. 1981).

Another enzymatic pathway leading to the formation of adenosine in heart cells is the methylation pathway (SAM pathway) (Daly 1982; Schrader 1983). Biological methylations by S-adenosylmethionine (SAM) lead to the production of S-adenosylhomocysteine (SAH), which is converted to adenosine and homocysteine by the enzyme S-adenosylhomocysteinase (SAH hydrolase). The SAM pathway, in the presence of homocysteine, can serve to maintain low levels of intracellular adenosine. Although the equilibrium constant for this reaction favours the formation of SAH, under physiological conditions it functions in the

hydrolytic direction, and is not greatly inhibited by the cytosolic environment (De La Haba and Cantoni 1959; Schrader 1983). Under physiological conditions (low adenosine and homocysteine levels), adenosine is continuously formed by the hydrolysis of SAH, at a rate of greater than 600 pmol/min/gm in guinea pig heart (Schrader 1983). However, since the basal release of adenosine into the coronary effluent perfusate of these hearts was only found to be 30-50 pmol/min/gm, it is obvious that substantial transformation of adenosine was occuring.

This metabolism of adenosine is catalysed by two enzymes in the cytosol of cells. Adenosine is phosphorylated to 5-'AMP by adenosine kinase, or deaminated to inosine by adenosine deaminase. Adenosine kinase has been observed to be inhibited by adenosine concentrations greater than 2.5 μM (Palella *et al*. 1980). In addition, it is inhibited by free ATP, free magnesium, ADP, AMP and SAH. The Km values for adenosine kinase in guinea pig and rat hearts are quite low (0.9 μM and 0.25 μM, respectively; Schrader 1983; Bowditch *et al*. 1985a), therefore it is believed that under physiological conditions the enzyme is functioning at saturation (Arch and Newsholme 1978; Schrader 1983). The Km value for adenosine deaminase (90μM) is much higher than that of adenosine kinase; therefore, under physiological conditions (low adenosine levels) phosphorylation will be favoured over deamination (Schrader 1983).

There is evidence that as much as 37 % of total myocardial adenosine is bound to SAH hydrolase (Schrader 1983; Ueland 1983). However, the major physiological fate of adenosine seems to be a recycling through 5'-AMP to ADP to ATP to SAM to SAH to adenosine. Adenosine kinase and adenosine deaminase serve to maintain low adenosine levels inside cells and to regulate the size of the cellular nucleotide pool (Plunkett and Cohen 1977; Daly 1982). Inhibition of adenosine kinase (with 5'-iodotubercidin) and/or adenosine deaminase (with deoxycoformycin) would lead to an increase in intracellular adenosine. Likewise, doubling the rates of adenosine formation by 5'-nucleotidase and SAH hydrolase would result in an 12-fold increase in adenosine levels (Schrader 1983).

The metabolic conversion of nucleosides inside cells is of significant importance in studies measuring nucleoside fluxes. Adenosine is said to be metabolically trapped within cells when it is converted by adenosine kinase to nucleotides (Plunkett and Cohen 1977; Plagemann and Wohlhueter 1980; Plagemann and Wohlhueter 1983). ATP is evidently impermeable to membranes (Plunkett and Cohen 1977; Bowditch *et al.* 1985b); however, some have presented evidence to the contrary (Chaudry *et al.* 1985). Nevertheless, the conversion to nucleotides inside cells tends to draw nucleosides into cells to levels above those expected if equilibration of unchanged nucleoside with extracellular concentrations were reached. In addition, as

intracellular adenosine concentrations increase, adenosine deaminase becomes more active. Conversion of adenosine to inosine may also increase the rate at which adenosine is taken up by preventing the buildup of adenosine concentrations inside cells. Inosine, which is also a substrate for the nucleoside transport system, may also be effluxing from cells as its concentration gradient increases. It is therefore obvious that metabolic conversion must be considered when nucleoside fluxes are being measured.

D. Nucleoside Transport

Since adenosine metabolism occurs mainly intracellularly, adenosine must be removed from its extracellular site of action in order to be inactivated. Conversely, the uptake of cytotoxic nucleoside analogues is necessary for "toxification", if the biological effects of these drugs or their metabolites occur within cells. This is accomplished by a membrane-located nucleoside specific transport system (Paterson *et al.* 1981; Paterson *et al.* 1983).

Many studies claiming to measure nucleoside transport (Bowditch *et al.* 1985a) have not measured flux rates of the transport process itself, but rather, the net accumulation and retention of nucleosides and their metabolic products. By definition, "uptake" studies measure the net cellular accumulation of nucleosides and their metabolites. It

includes both "transport" and intracellular metabolism. Transport is the initial step in the uptake process, before metabolism of the permeant and counter fluxes become involved. Estimates of the rate of nucleoside "transport" require the measurement of the "initial rate" of nucleoside flux (Plagemann and Wohlhueter 1980; Paterson *et al*. 1981; Paterson *et al*. 1983; Plagemann and Wohlhueter 1983). This thesis will be consistent with the use of the terms "uptake" and "transport" as described above.

The transport of nucleosides is often very rapid and equilibration of the intracellular and extracellular permeant concentrations may occur within a few seconds (Paterson *et al*. 1981; Plagemann and Wohlhueter 1983). As equilibration is reached, the rate of nucleoside uptake becomes equal to the rate of intracellular metabolism of the permeant (Plagemann and Wohlhueter 1980). Therefore, it is often necessary to measure nucleoside flux rates after very short incubation intervals, utilizing rapid sampling techniques (Paterson *et al*. 1983; Plagemann and Wohlhueter 1983), to ensure that initial rates are defined, i.e. an approximation of the rate of nucleoside flux at zero seconds of incubation (Paterson *et al*. 1981). Many studies have chosen time intervals when intracellular permeant concentration is less than 20 % of extracellular concentration to ensure that initial rates of uptake are measured (Paterson *et al*. 1981; Jarvis *et al*. 1982a; Jarvis *et al*. 1983a). In addition, because short incubation

intervals are often needed for initial rate transport studies, isolated cell preparations devoid of tissue structure are necessary to ensure that diffusion of the permeant to its site of entry is not rate-limiting in the influx process. Another methodological approach to separating transport events from metabolic events has been the measurement of nucleoside fluxes in cells intrinsically lacking appropriate permeant kinase activity, for example, uridine and thymidine fluxes in human erythrocytes (Oliver and Paterson 1971), or in cells rendered incapable of permeant phosphorylation by mutational deletion of appropriate kinases or by ATP-depletion (Wohlhueter *et al.* 1979; Paterson *et al.* 1981). In addition to using nonphosphorylating cell systems to separate the transport and metabolic components of nucleoside uptake, researchers have utilized nonphosphorylated permeants such as 5'-deoxyadenosine (Kessel, 1978).

Nucleoside transport has been studied in a number of isolated cell systems including erythrocytes (Oliver and Paterson 1971; Cass *et al.* 1974; Jarvis *et al.* 1982a; Jarvis *et al.* 1983a; Plagemann and Wohlhueter 1984a), various cultured cells (Kessel 1978; Cass *et al.* 1981; Harley *et al.* 1982; Belt 1983; Chello *et al.* 1983; Paterson *et al.* 1984; Plagemann and Wohlhueter 1984b) and lymphocytes (Wierda and Pazdernik 1981). In several systems where initial rates of nucleoside uptake could be measured in the absence of metabolic interactions, nucleoside permeation was found to

be nonconcentrative and saturable; that is, nucleoside transport occured by a facilitated diffusion system (Oliver and Paterson 1971; Paterson *et al.* 1981; Plagemann and Wohlhueter 1983). It has been shown to be inhibitable by a wide variety of compounds, such as NBMPR, dipyridamole, dilazep and some benzodiazepines, such as diazepam (Paterson *et al.* 1980; Paterson *et al.* 1983). Nucleoside transport by erythrocytes and various cultured cells has been described by a simple carrier model (Plagemann and Wohlhueter 1980; Jarvis *et al.* 1983a; Plagemann and Wohlhueter 1983) mediated by a single type of carrier which possesses a broad specificity. The transporter accepts as substrates a wide variety of purine and pyrimidine nucleosides, although with differing affinities (Oliver and Paterson 1971; Berlin and Oliver 1975; Plageman and Wohlhueter 1980).

Three experimental protocols have been utilized to study the transport of nucleosides and the functional symmetry of the transport process: zero-*trans* influx, zero-*trans* efflux and equilibrium exchange (Plagemann and Wohlhueter 1983). In zero-*trans* entry, the flux of various concentrations of radiolabelled substrate by cells is measured when the intracellular concentration of permeant is initially zero. In zero-*trans* efflux protocols, cells are preloaded with various concentrations of radiolabelled substrate, and its flux into the extracellular medium initially free of substrate is measured. Finally, in equilibrium exchange experiments, the movement of

radiolabelled substrate in either direction is measured when the initial intra- and extracellular concentrations are the same. In both fresh erythrocytes and various cultured cells, rates of zero-*trans* influx and zero-*trans* efflux have been found to be similar, indicating that the nucleoside carrier exhibits directional symmetry (Wohlhueter and Plagemann 1982; Jarvis *et al*. 1983a; Plagemann and Wohlhueter 1983; Plagemann and Wohlhueter, 1984a). However, nucleoside transporters on erythrocytes from outdated blood possess directional asymmetry, with zero-*trans* efflux velocities greater than zero-*trans* influx (Jarvis *et al*. 1983a). In addition, the mobility of the empty and loaded nucleoside carriers are equal in most cultured cells, since the rate of zero-*trans* flux is equal to the rate of equilibrium exchange flux (Wohlhueter and Plagemann 1982; Plagemann and Wohlhueter 1983). In contrast, in erythrocytes the rate of equilibrium exchange is greater than the rate of zero-*trans* flux, indicating that the loaded carrier moves faster than the unloaded carrier (Wohlhueter and Plagemann 1982; Jarvis *et al*. 1983a; Plagemann and Wohlhueter 1983). The term "*trans*-stimulation" (or accelerated exchange diffusion) has been used to describe the effect of substrate on both sides of the membrane on permeant flux in erythrocytes (Pickard and Paterson 1972; Cass and Paterson 1972; Paterson *et al*. 1981; Plagemann and Wohlhueter 1983).

The nucleoside transport system is dependent on a number of physiological variables. It has been shown to be temperature dependent, since the Km and Vmax values for the transport process increase as temperature is increased (Berlin 1973; Kolassa *et al*. 1978; Wohlhueter *et al*. 1979; Plagamann and Wohlhueter 1984a). Kolassa and associates (1978) observed a 3- to 5-fold variation in Km and Vmax values for adenosine influx in human erythrocytes with changes in pH from 7 to 8. Finally, calcium, magnesium, sodium and potassium ions had no apparent effect on adenosine transport in human erythrocytes, while the absence of chloride ions decreased the rate of adenosine influx almost 2-fold in the same system (Ford *et al*. 1985).

Rates of nucleoside transport have not been measured in heart tissue. One group of researchers have claimed to have measured rates of adenosine transport in rat cardiac myocytes (Bowditch *et al*. 1985a). However, the use of long incubation intervals, the absence of measurement of non-mediated influx in the presence of a specific transport inhibitor, and the fact that the Km values for adenosine transport and adenosine kinase activity did not differ significantly, would indicate that they were measuring total accumulation rates. Nucleoside "uptake" has been studied in various heart preparations (Hopkins and Goldie 1971; Olsson et al. 1972; Mustafa 1979; Barker and Clanachan 1982; Williams *et al*. 1984). Although initial rates of nucleoside transport have not been measured directly in cardiac tissue,

the nucleoside transport system has been studied indirectly in heart with the use of nitrobenzylthioinosine (NBMPR), a high affinity binding probe (Williams *et al*. 1984).

E. Inhibition of Nucleoside Transport

NBMPR as a High Affinity Binding Probe

The search for compounds which could affect nucleoside metabolism, and have potential therapeutic usefulness in cancer chemotherapy, lead to the development of the S'-substituted 6-thiopurine derivative nitrobenzylthioinosine (NBMPR; Henderson *et al*. 1972; Paul *et al*. 1975). NBMPR and other S'-6-thiopurine derivatives protect various cultured cells against the cytotoxic actions of several nucleoside analogues which produce their effects intracellularly (Henderson *et al*. 1972; Paterson *et al*. 1979a; Paterson et al., 1979b). Therefore, in many systems, nucleosides enter cells via an NBMPR-sensitive transport system (exceptions will be discussed in the heterogeneity section of this Introduction). NBMPR does not appear to inhibit intracellular metabolism of nucleosides since it had no effect on adenosine kinase activity up to μM concentrations (Olsson *et al*. 1972; Cass and Paterson 1977; Paterson *et al*. 1977) and it inhibits the transport of the nonmetabolizable permeants, uridine and thymidine, into human erythrocytes (Cass and Paterson 1972). In systems where initial rates of nucleoside flux could be measured,

NBMPR and several S'-6-thiopurine derivatives have been found to be potent, selective inhibitors of nucleoside transport (Oliver and Paterson 1971; Paul *et al.* 1975; Rogler-Brown and Parks 1980).

In systems where nucleoside transport cannot yet be measured, such as intact heart tissue and brain, tritiated NBMPR ([^{3}H]NBMPR) has been used as a binding probe for studying the nucleoside transport complex. This ligand binds with high affinity (Kd between 0.01 and 1 nM), but reversibly, to specific sites in most tissues, including: membrane preparations from heart (Williams and Clanachan 1983; Williams *et al.* 1984), CNS (Hammond and Clanachan 1983; Geiger and Nagy 1984; Hammond and Clanachan 1984a; Hammond and Clanachan 1984b; Hammond and Clanachan 1985; Nagy *et al.* 1985; Verma and Marangos 1985), various cultured cells (Lauzon and Paterson 1977; Wohlhueter *et al.* 1978; Paterson *et al.* 1980; Dahlig-Harley *et al.* 1981; Koren *et al.* 1983) and erythrocyte membranes (Cass *et al.* 1985), as well as intact erythrocytes (Hammond *et al.* 1981; Jarvis *et al.* 1982a; Jarvis *et al.* 1983a; Jarvis *et al.* 1983b).

There is very strong evidence that NBMPR binding sites are inhibitory sites on (or associated with) the nucleoside transport complex. Much of this comes from work using intact cell preparations (such as erythrocytes and various cultured cell lines) where both NBMPR binding and initial rate transport velocities may be measured.

(1) Cass and associates (1974) showed that

inhibition of uridine transport into human erythrocytes was proportional to the number of high affinity sites occupied by NBMPR.

(2) In erythrocytes from many species, the maximum NBMPR site density (Bmax) was found to be proportional to the maximum velocity of uridine transport (Jarvis *et al*. 1982b). Assuming that one molecule of NBMPR binds to one high affinity site, the translocation capacity for uridine (140 - 180 molecules/site/sec at 25°C; Jarvis and Young 1982) was found to be similar for transporters from all species of erythrocytes. That is, in erythrocytes, the ability of cells to transport nucleosides is a function of the number of functional transporters, as indicated by the number of NBMPR sites (Jarvis and Young 1982). Nucleoside-impermeable sheep erythrocytes possess no detectable uridine transport or NBMPR binding capacity (Jarvis *et al*. 1982; Jarvis and Young, 1982). Similarly, the AE, clone of S49 mouse lymphoma cells in which the ability to transport nucleosides has been lost by mutation (and thus, become resistant to cytotoxic nucleosides), also no longer possess demonstrable high affinity NBMPR binding sites (Cass *et al*. 1981).

(3) The inhibition constant obtained for NBMPR-mediated inhibition of uridine transport in

human erythrocytes was the same as the Kd for NBMPR binding to its high affinity sites (Jarvis *et al.* 1982a). Similarly, inhibition constants obtained for benzodiazepine-mediated inhibition of NBMPR binding and inhibition of nucleoside transport in human erythrocytes were similar (Hammond *et al.* 1983).

(4) Photoaffinity labelling of [^{3}H]NBMPR binding sites in erythrocytes (Jarvis *et al.* 1983c; Wu *et al.* 1983; Young *et al.* 1983), guinea pig brain (Jarvis and Ng 1985), rat and guinea pig liver (Wu and Young 1984), guinea pig heart (Kwan and Jarvis 1984) and cultured mouse lymphoma cells (Almeida *et al.* 1984; Young *et al.* 1984) indicate that NBMPR is binding to a band 4.5 polypeptide associated with the nucleoside transporter, with an apparent molecular weight of 45,000-66,000. Also, it is possible to reconstitute inhibitable nucleoside transport activity into liposomes using either a crude human erythrocyte membrane extract or the partially purified band 4.5 preparation which binds NBMPR (Belt *et al.* 1984; Tse *et al.* 1985).

The results presented above provide overwhelming evidence that NBMPR binds to sites associated with the nucleoside transport system in erythrocytes and several types of cultured cells, and that occupation of these sites leads to inhibition of nucleoside transport. This is not to

deny the existence of nucleoside transporters that are of low sensitivity to inhibition by NBMPR (as will be discussed subsequently). However, NBMPR binding could be a useful probe for studying the nucleoside transport system in most tissues preparations (for example, heart and brain) where transport rates are difficult to measure.

There is much controversy as to how high affinity NBMPR binding sites and the permeation site of the nucleoside transporter interact. It is still unknown whether NBMPR binds to the permeation site itself, or to a separate site linked in some way to the transporter by an allosteric interaction. Reconstitution experiments with partially purified NBMPR binding proteins (Belt *et al.* 1984) indicates a close association between these sites. Many nucleosides are also competitive inhibitors of NBMPR binding (Cass and Paterson 1976; Paterson *et al.* 1983). Several nucleosides, including adenosine, inosine, thymidine, guanosine, uridine and cytidine, possessed inhibition constants (Ki values) for inhibition of NBMPR binding that are similar to their Michaelis Menten constants (Km values) for transport into cultured cells and human and sheep erythrocytes (Berlin and Oliver 1975; Jarvis *et al.* 1982a and 1982b; Plagemann and Richey 1974; Paterson 1979; Chello *et al.* 1983). This can be interpreted as evidence that NBMPR causes nucleoside transport inhibition by binding to the nucleoside permeation site. Although NBMPR is structurally related to nucleoside permeants, many agents known to inhibit competitively NBMPR

binding (such as dipyridamole, dilazep and diazepam) are structurally unrelated to nucleosides (Paterson *et al.* 1983). However, recent structural analysis (Codding and Jakana, personal communication) has revealed that the spatial orientation of functional groups on dipyridamole and adenosine are quite similar.

Recent studies have investigated the effects of various transport inhibitors and nucleoside permeants on the kinetics of dissociation of [^{3}H]NBMPR from its high affinity sites on human erythrocyte membranes (Jarvis *et al.* 1983d) and cultured hamster fibroblasts (Koren *et al.* 1983). When dissociation assays were conducted in the presence of a high concentration of unlabelled NBMPR (1000-fold greater than its Kd to ensure that binding site "reassociation" was negligible, it was assumed that rates of dissociation of [^{3}H]NBMPR would be unaltered in the presence of agents that bound only to the same site as [^{3}H]NBMPR (Koren *et al.* 1983). It was found that dissociation rates were increased in the presence of adenosine or uridine and decreased in the presence of dipyridamole and dilazep. Although the possibility was not ruled out that NBMPR sites and the permeant sites were the same, these results have been interpreted as evidence that the nucleoside permeants bind to a site separate from the NBMPR site, indicating that the nucleoside permeant site and the inhibitory site are separate, but somehow allosterically connected. In addition, it was proposed that dilazep and dipyridamole do

not bind to the same site as NBMPR, but rather to closely associated sites on the transporter. However, it should be noted that at their Ki concentrations for competitive inhibition of NBMPR binding neither adenosine, uridine, dipyridamole nor dilazep significantly altered the [^{3}H]NBMPR dissociation rate (Jarvis *et al.* 1983b; Koren *et al.* 1983). Furthermore, decreases in dissociation rates by dipyridamole were only observed at concentrations 100-fold greater than its apparent Ki value for competitive inhibition of NBMPR binding. It is possible that the high concentrations necessary to elicit changes in dissociation rates could have uncovered nonspecific effects of these agents which have overshadowed the competitive nature of their binding to NBMPR sites. Nevertheless, the question of whether NBMPR binding sites and the nucleoside permeation site are similar, with a second hydrophobic binding site to accommodate the nitrobenzyl ring of NBMPR (Jarvis and Young 1982), or distinct, but functionally associated sites, remains to be answered.

It is believed that NBMPR binds to the external surface of cell membranes (Jarvis *et al.* 1982b; Jarvis and Young 1982). NBMPR inhibited zero-*trans* uridine influx into nucleoside-permeant sheep erythrocytes in a competitive manner (apparent Ki, 1 nM). However, NBMPR was a noncompetitive inhibitor of zero-*trans* efflux of uridine (apparent Ki of 1.5 nM) from the same cells (Jarvis *et al.* 1982b). Possibly the hydrophobic binding site for the

nitrobenzyl ring of NBMPR is only present on the external membrane (Jarvis and Young 1982). In addition, the nucleoside transporter is believed to exhibit chemical asymmetry since p-chloromercuriphenylsulphonate and trypsin disrupted NBMPR binding and uridine transport only when the inner membrane was exposed (Jarvis and Young 1982). With these facts in mind, a model has been proposed for the nucleoside transporter (Jarvis and Young 1982), consisting of a substrate binding site and two conformations of free carrier: one exposed to the external surface of the membrane and the other exposed to the internal surface. Transport depends on the interconversion of the two conformational forms (Jarvis and Young 1982). Irrespective of how NBMPR sites are physically associated with the nucleoside tranport system, this compound may be a useful probe for studying the transport mechanism and potential heterogeneity of transport systems.

Coronary Vasodilators

A number of so-called "coronary vasodilators" have been found to be inhibitors of nucleoside transport. Dipyridamole was introduced clinically for the treatment of angina (Charlier 1966). It has been shown to dilate both the coronary resistance vessels (Afonso 1970) and large coronary arteries (Hintze *et al.* 1983). Dilazep has also been shown to possess coronary vasodilatory activity (Sano *et al.* 1972). The vasoactivity of the coronary

vasodilators, such as dipyridamole and dilazep is attributed to the potentiation of adenosine-induced vasodilation, following inhibition of nucleoside transport by these agents (Kalsner 1975; Berne *et al.* 1983). However, these drugs are no longer used for their coronary vasodilatory properties because of the phenomenon of "coronary steal".

Both dipyridamole and dilazep inhibited the uptake of adenosine into guinea pig heart (Hopkins and Goldie 1971; Hopkins 1973; Williams *et al.* 1984), canine heart (Olsson *et al.* 1972) and cultured chick embryo heart cells (Mustafa 1979). Although inhibition of the nucleoside transport process has not yet been measured directly in heart, it has been studied indirectly using [^{3}H]NBMPR binding techniques. A number of coronary vasodilators such as dipyridamole, dilazep and hexobendine (which are structurally unrelated to nucleosides) are competitive inhibitors of [^{3}H]NBMPR binding (Williams *et al.* 1984). Studies using erythrocytes indicate that the coronary vasodilators inhibit nucleoside transport by interacting with NBMPR sites (Paterson *et al.* 1980).

Benzodiazepines

A number of benzodiazepines, including diazepam, are inhibitors of nucleoside transport. Benzodiazepines inhibited the transport of uridine into human erythrocytes (Hammond *et al.* 1983). In addition, these agents inhibited the accumulation of adenosine into tissue preparations from guinea pig heart (Barker and Clanachan 1982; Williams *et al.*

1984) and brain (Davies and Hambley 1983). Benzodiazepines inhibited NBMPR binding in a competitive manner in preparations from heart (Williams *et al.* 1984) and brain (Hammond *et al.* 1981). Some researchers have proposed that benzodiazepines produce their central depressant effects via inhibition of adenosine transport, thereby potentiating the depressant effects of adenosine (Phillis *et al.* 1981). However, the concentrations at which benzodiazepines which produce their anxiolytic, anticonvulsant and sedative effects are much lower than those that inhibit nucleoside transport (Hammond and Clanachan 1984a; Mohler and Okaka 1978). In addition, the order of affinity of benzodiazepines for NBMPR sites in guinea pig CNS membranes (Hammond and Clanachan 1984a) differ significantly from their order of affinity for "high affinity" benzodiazepine recognition sites (Mohler and Okada 1978).

Potentiation of Nucleoside Action

Since adenosine is removed from the extracellular compartment by the nucleoside transport system, agents that block its transport would be expected to potentiate the effects of adenosine at its extracellular receptors. Potentiation of exogenously applied or endogenously released adenosine by nucleoside transport inhibitors such as NBMPR, hydroxynitrobenzylthioguanosine (HNBTG), dipyridamole, dilazep, hexobendine or diazepam have been reported in preparations of guinea pig right atria (Hopkins 1973),

guinea pig left atria (Clanachan and Marshall 1980; Moritoki *et al*. 1985), guinea pig taenia caeci (Baer 1983; Tonini *et al*. 1983), guinea pig taenia coli (Moritoki *et al*. 1985) and rat vas deferens (Muller and Paton, 1979; Clanachan and Marshall 1980; Clanachan and Muller 1980).

NBMPR sites are distinct from adenosine receptors, since the adenosine receptor antagonist, theophylline, did not inhibit NBMPR binding (Hammond and Clanachan 1984a). Inhibitors of nucleoside transport do not inhibit the binding of adenosine to its receptors (Mustafa 1980; Marangos 1984; Verma and Marangos 1985). In addition, the regional distribution of NBMPR sites in the CNS (Hammond and Clanachan 1983) differs from that of adenosine receptors (Williams and Risley 1980). Therefore, it is clear that inhibitors of nucleoside transport do not potentiate nucleoside actions by interacting directly with adenosine receptors.

The adenosine analogue, 2-chloroadenosine, has been found to be a more potent agonist at adenosine receptors than adenosine (Muller and Paton 1979; Clanachan and Marshall 1980; Clanachan and Muller 1980; Baer 1983). However, nucleoside transport inhibitors have not been demonstrated to potentiate the effects of 2-chloroadenosine in preparations in which the effects of adenosine are potentiated (Muller and Paton 1979; Clanachan and Marshall 1980; Clanachan and Muller 1980; Baer 1983; Moritoki *et al*. 1985). This has led some to conclude that 2-chloroadenosine

in not a substrate for the nucleoside transport system (Muller and Paton 1979), without ever having directly measured the transport capacity of cells for this analogue. Recently, 2-chloroadenosine has been found to be a substrate for the nucleoside transport system in erythrocytes (Jarvis *et al.* 1985). This finding would appear to raise doubts concerning the mechanism of nucleoside transport inhibitor-induced potentiation of adenosine actions.

F. Nucleoside Transport System Heterogeneity

There has been increasing evidence for the existence of nucleoside transport heterogeneity. These differences take a number of forms, including, species and tissue variability with respect to nucleoside transport site density, as well as differing susceptibilities to inhibition by various known nucleoside transport inhibitors. NBMPR binding studies in a number of tissues have shown that species differences exist for the affinity of various inhibitors for these sites (with much of this work concentrating on the differences between guinea pig and rat tissue), and this may be interpreted within the context of transport inhibition. In addition, there is an increasing volume of literature which gives evidence for NBMPR-insensitive transporters and NBMPR-insensitive components of nucleoside transport in various cultured cells. These are characteristics which may be exploited in cancer chemotherapy.

NBMPR Site Density

In a thorough investigation of nucleoside transport activity in erythrocytes from a variety of species, Jarvis and associates (1982a) have shown that species differences exist with respect to high affinity NBMPR binding site density. For example, Bmax values in dog, guinea pig, rat, rabbit, and human erythrocytes were 0, 27, 300, 9,000 and 11,000 molecules/cell, respectively. These differences in NBMPR site density directly correlated with the maximum velocities of uridine transport in these cells (Jarvis *et al*. 1982a).

In tissues where initial rates of nucleoside flux have not been measured, such as heart (Williams *et al*. 1984), brain (Hammond and Clanachan 1984b; Hammond and Clanachan 1985; Verma and Marangos 1985) and lung (Shi *et al*. 1984), species differences in NBMPR site density have also been found. In membrane preparations from ventricular heart tissue, NBMPR site density varies from 195 fmol/mg protein in rat to 1700 fmol/mg protein in guinea pig, and as high as 2036 fmol/mg protein in membranes from bovine left ventricle (Williams *et al*. 1984). In addition, in guinea pig (Hammond and Clanachan 1983) and rat (Geiger and Nagy 1984) brain, NBMPR binding has been shown to possess differences in regional distribution. Since nucleoside transport has not yet been measured in heart, brain and lung, it is unknown whether NBMPR site density may be predictive of nucleoside transport capacity (a question

addressed in this thesis), as has been shown in erythrocytes (Jarvis *et al.* 1982a).

Drug Affinity for NBMPR Sites

Species differences exist with respect to drug affinity for NBMPR sites in a number of tissues, including brain (Hammond and Clanachan 1985; Verma and Marangos 1985), heart (Williams *et al.* 1984) and lung (Shi *et al.* 1984). Cortical membranes from rat and mouse possess only one type of NBMPR binding site, which shows a low affinity for dipyridamole (Hammond and Clanachan 1985). Dipyridamole was a more potent inhibitor of NBMPR binding to cortical membranes from guinea pig (Ki = 11 nM), than rat (Ki = 404 nM). In contrast to NBMPR, dipyridamole produced a biphasic profile of inhibition of NBMPR binding to cortical membrane preparations from rabbit and dog (Hammond and Clanachan 1985). These species possess sites with a high affinity (IC_{50} less than 29 nM) and sites with "rat-like" low affinity for dipyridamole (IC_{50} greater than 5 μM). Rabbit cortical membranes possess high and low affinity NBMPR sites, with Kd values of 0.4 nM and 13.8 nM, respectively. The binding sites for NBMPR in guinea pig cortical membranes appear to be similar to those sites on human erythrocytes (Hammond and Clanachan 1982; Hammond and Clanachan 1984a; Hammond and Clanachan 1985), since both possess similar affinities for various known transport inhibitors, such as dipyridamole, dilazep and hexobendine (Hammond 1983; Hammond

and Clanachan, in press). These results point to the existence of heterogeneous NBMPR sites in brain tissue.

There is also evidence that heterogeneous NBMPR sites are present in heart (Williams *et al*. 1984) and lung tissue (Shi *et al*. 1984). Dipyridamole, dilazep, hexobendine and lidoflazine inhibited competitively the binding of NBMPR to ventricular membranes from guinea pig (Ki values of 9.83, 0.66, 11.96, and 606 nM, respectively) with higher potency than to rat (Ki values of 276, 3.49, 129, and 5745 nM, respectively). A similar lower affinity of dipyridamole for NBMPR sites in rat, as compared to guinea pig, was also oberved in membranes from lung tissue (Shi *et al*. 1984). Conversely, adenosine had a higher affinity for NBMPR sites in rat cardiac membranes (Ki of 63.7 μM) as compared to guinea pig (Ki of 200.9 μM), while other nucleosides, such as uridine and 2-chloroadenosine, did not show species differences with respect to competitive inhibition of NBMPR binding (Williams *et al*. 1984). The evidence presented shows the existence of heterogeneous NBMPR sites, with sites in rat brain, heart and lung possessing a much lower affinity for various inhibitors, as compared to sites in these tissues from guinea pig. Guinea pig NBMPR sites demonstrate similarities to NBMPR sites on human erythrocytes (Hammond and Clanachan 1984a; Hammond and Clanachan 1985). In addition, membrane preparations from guinea pig and human brain show similar NBMPR binding characteristics (Marangos 1984; Verma and Marangos 1985).

The question remains as to how differences in the affinity of various agents for NBMPR binding sites relate to their ability to inhibit nucleoside transport in these tissues.

There is preliminary evidence that the nucleoside transport system in rat possesses a lower susceptibility to inhibition by a number of recognized inhibitors of nucleoside transport, than guinea pig. In guinea pig heart, the transport inhibitors had an order of affinity (dilazep > hydroxynitrobenzylthioinosine > dipyridamole > hexobendine >> lidoflazine >> diazepam) for NBMPR sites which was similar to their order of potency as inhibitors of adenosine accumulation and as potentiators of the negative inotropic actions of adenosine (Williams *et al.* 1984). This constitutes preliminary evidence that NBMPR binding results in inhibition of nucleoside transport in heart. In contrast, various inhibitors, including dipyridamole, showed a much lower potency in rat heart to produce these effects (Williams *et al.* 1984). The IC_{50} values for dipyridamole-mediated inhibition of adenosine "uptake" into rat and guinea pig ventricular tissue were > 10 μM and 0.1 μM, respectively (Williams *et al.* 1984). In addition, 10 μM dipyridamole produced no significant potentiation of the negative inotropic actions of adenosine on electrically driven rat left atria, while 0.1 μM dipyridamole had a significant potentiating effect in guinea pig (Williams *et al.* 1984). The lower potency of dipyridamole to inhibit adenosine "uptake" into rat in comparison with guinea pig

tissue has been well documented (see Hammond and Clanachan 1985) in heart (Hopkins and Goldie 1971; Kolassa *et al.* 1971; Williams *et al.* 1984), brain (Davies and Hambley 1983), lung (Bakhle and Chelliah 1983) and erythrocytes (Kolassa and Pfleger 1975).

Guinea pig left ventricular tissue possesses a 6-fold greater nucleoside transport dependent accumulation of [^{3}H]adenosine than rat heart (Williams *et al.* 1984), which is consistent with the larger NBMPR site density (Bmax) in guinea pig heart. Although nucleoside transporters in rat heart, brain and lung appear to be different from those in guinea pig, it is not known whether the lower potency of agents, such as dipyridamole, in rat is due to transporter heterogeneity or the inability to detect small inhibitor-induced changes in transport because of the apparent lower transport activity in rat.

NBMPR-Insensitive Nucleoside Transport

Heterogeneity exists amongst nucleoside transport systems from a variety of cultured mammalian neoplastic cells. While S49 mouse T lymphoma cells have only transporters that are sensitive to inhibition by NBMPR, a number of cell lines, including Walker 256 rat carcinosarcoma and Novikoff rat hepatoma cells, possess facilitated transport systems for nucleosides that are not inhibited by low concentrations of NBMPR, since these cells lack high affinity NBMPR binding sites (Belt 1983; Paterson

et al. 1983; Plagemann and Wohlhueter 1984c). Furthermore, cell lines, such as L1210 mouse leukemia cells, P388 cells and HeLa cells, possess both NBMPR-sensitive and NBMPR-insensitive nucleoside transport (Lauzon and Paterson 1977; Belt 1983; Plagemann and Wohlhueter 1984c). Lauzon and Paterson (1977) found that in HeLa cells approximately 25-30 % of the total "uptake" capacity was not inhibited by 5 nM NBMPR, a concentration that saturated high affinity NBMPR sites in these cells. This remaining component was inhibited by a higher concentration of NBMPR (5 μM; Lauzon and Paterson 1977). In P388 cells, > 7 % of uridine transport was resistant to 100 nM NBMPR, but was inhibited by further addition of 20 μM dipyridamole (Plagemann and Wohlhueter 1984c). In L1210 cells, 20 % of transport was resistant to 1 μM NBMPR, but was still distinguishable from passive diffusion or a second transport mechanism (Belt 1983). The size of this component in L1210 cells was large enough to render the "uptake" of nucleosides virtually insensitive to NBMPR over prolonged periods of time (10 min; Belt 1983).

Two selected clones of S49 cells, KAB1 and KAB5, have been found to possess NBMPR-insensitive nucleoside transport (Aronow *et al.* 1985). The KAB5 cell line is 70-75 % deficient in NBMPR binding sites, which is consistent with its loss in sensitivity to NBMPR. However, KAB1 cells possess high affinity NBMPR binding comparable to that in the wild type S49 cells, but this does not appear to be

linked to inhibition of nucleoside transport. This is evidence that NBMPR binding sites and the nucleoside carrier sites are genetically distinguishable, and therefore, possibly separate sites (Aronow *et al*. 1985).

Another form of nucleoside transport has been found to exist in brush border membranes of epithelial cells of the kidney and the intestines. A sodium-dependent transport system has been found which is not inhibited by NBMPR (Jarvis personal communication).

The above mentioned studies have demonstrated the existence of heterogeneous types of nucleoside transport systems which have differing susceptibility to inhibition by NBMPR. Since many cancer cells may possess NBMPR-insensitive nucleoside transport, NBMPR may have therapeutic potential as a pre-treatment to protect host cells from the effects of cytotoxic nucleoside analogues used in cancer chemotherapeutic regimens (Paterson *et al*. 1983). The significance of heterogeneous nucleoside transport in heart will by discussed subsequently.

G. Rationale

The introduction described the important regulatory functions of adenosine in the heart and other tissues. It is known that adenosine acts at receptors located on the extracellular surface of cells. The nucleoside transport system is vital in the regulation of adenosine concentration in the vicinity of these receptors. This transport system

permits the release of adenosine from cells, and also controls the rate of removal of adenosine from the extracellular space. Furthermore, drug-induced inhibition of nucleoside transport may influence extracellular adenosine levels.

Studies hitherto performed on the fluxes of nucleosides across cardiac membranes and on drug-induced inhibition of these fluxes have failed to distinguish between transport *per se* and the accumulation of permeant. Although significant information about the nucleoside transport process comes from studies using erythrocytes or cultured cells, to date no study has investigated the transport of adenosine or other nucleosides into cardiac cells. Attempts to study the transporter by indirect means have utilized NBMPR as a high affinity binding probe, but these have been based on the assumption that the nucleoside transport complex in heart is similar to that in erythrocytes (ie. NBMPR binding site occupancy results in inhibition of nucleoside transport). In order to measure the transport of nucleosides into cardiac cells, a dissociated cell preparation is required. Dissociated ventricular myocytes from guinea pig and rat heart were prepared and used for the present study. With this preparation, the following topics could be investigated:

(1) [^{3}H]NBMPR binding characteristics,

(2) drug-induced inhibition of [^{3}H]NBMPR binding,

(3) nucleoside uptake and transport, and

(4) drug-induced inhibition of nucleoside transport.

These studies were designed to answer the following questions regarding the myocyte nucleoside transport system:

(1) does NBMPR bind to inhibitory sites associated with the nucleoside transport system? This was determined by comparing drug potencies for inhibition of NBMPR binding and nucleoside transport,

(2) do species differences exist with respect to drug-induced inhibition of both NBMPR binding and nucleoside transport? Comparisons were made between guinea pig and rat myocyte preparations,

(3) do nucleosides display similar affinities for NBMPR sites and the transport process? The affinities of adenosine and 2-chloroadenosine for high affinity NBMPR sites were compared with their affinities for the transport process.

The answers to these questions will indicate whether NBMPR is a useful, high affinity probe for the cardiac nucleoside transport system and will explore potential heterogeneities among transporters. Heterogeneity would indicate that differences in susceptibility to nucleoside transport inhibitors exist. This may lead to the development of drugs that may modify selectively the cardiac actions of adenosine.

II. METHODS

A. Tissue Preparation

Preparation of Cardiac Membranes

This method was used for both guinea pig and rat cardiac membrane preparations. Animals (200-250 g) were killed by decapitation and their hearts were removed rapidly and placed in ice-cold 0.32 M sucrose. Ventricular tissue was chopped into small (10 mg) segments, homogenized in 20 volumes (w/v) of ice-cold 0.32 M sucrose (Polytron, type PT 1020 350D, setting 5 for 20 s) and centrifuged at 1000 g for 10 min. The supernatant fraction was recentrifuged at 20,000 g for 20 min at 4°C producing a crude (P_2) membrane pellet. The P_2 membrane pellet was resuspended in 20-50 volumes of 50 mM Tris-HCl buffer, ph 7.4, to a protein concentration of 0.5 mg/ml. Protein concentration were determined by the Lowry method (Lowry et al. 1951). Experiments were performed using freshly prepared cardiac membranes.

Preparation of Ventricular Myocytes

This method was used for both guinea pig and rat ventricular myocyte preparation. Male animals (200-250 g) were heparinised (2.5 u/g, body weight) and killed by decapitation. Hearts were removed and placed in

carbogenated (95% CO_2, 5% O_2) Joklik medium (Appendix 1), pH 7.4, 37°C. The hearts were then perfused retrogradely via their aortas with Joklik medium, 37°C, 3-5 ml/min, for 2-4 min in order to wash out blood remaining in the coronary circulation. Perfusion (3-5 ml/min, at 37°C) was continued for 30 to 40 min with a collagenase enzyme mixture (Digestion medium, Appendix 1) containing 300 u/ml Sigma collagenase type V, 150 u/ml Sigma collagenase type IA and 0.1 % bovine serum albumin, prepared in Joklik medium. After removing the hearts from the perfusion apparatus, the ventricles were aggitated in disruption medium (Appendix 1), to allow the myocytes to dissociate from the remaining heart "chunk". Following mechanical dissociation, the ventricular myocytes were washed twice and resuspended in assay medium (modified Dulbecco's phosphate buffered saline, Appendix 1). Binding assays were conducted with 30,000 to 60,000 myocytes per assay and transport assays were conducted with 200,000 to 500,000 myocytes per assay. Cells were counted by Coulter Counter methodology. All experiments were performed using freshly prepared cardiac myocytes.

Cell viability was determined by the rod-shaped appearance and trypan blue (0.3 mM) exclusion of viable cells. Assays were conducted with preparations containing in excess of 70% viable cells. Mortality rates were determined by measuring cell viability at 30 min intervals over a period of 3 hours. Cell mortality rates were found to be < 5% per hour for both species (with data from 3 and 4

separate myocyte preparations from guinea pig and rat, respectively). Intracellular volumes were determined by subtracting the [^{14}C]sucrose space of the myocyte pellets (representing the extracellular space of the pellet) from the [^{3}H]water space of the pellets (representing the total space of the myocyte pellets). Intracellular space of both guinea pig and rat myocyte pellets were found to be approximately 10 µl per million cells.

B. [^{3}H]NBMPR Binding Assays with Cardiac Membranes

General Methodology

[^{3}H]NBMPR binding to cardiac membranes was evaluated as previously described by Williams *et al.* 1983. Equilibrium binding assays were initiated with the addition of 0.4 ml cardiac membrane suspension (containing 0.2 mg of protein) to mixtures containing appropriate concentrations of [^{3}H]NBMPR in the presence or absence of a specific inhibitor of nucleoside transport. All assays were conducted in a total volume of 1 ml in polypropylene microcentrifuge tubes (1.5 ml) at 22°C. Assay mixtures were incubated 20 min to obtain equilibrium binding of [^{3}H]NBMPR. The reaction was stopped by centrifugation (Eppendorf 5412 microcentrifuge for 2 min) to separate the free ligand from the membrane-associated [^{3}H]NBMPR. The membrane pellets were then washed once with additional ice-cold 50 mM Tris-HCl (1 ml). Membrane pellets were digested overnight with

250 µl of 0.5 N KOH and neutralized with 100 µl 1.25 N HCl. Aliquots (200 µM) were transfered to mini-scintillation vials (5 ml) and 5 ml of tritisol scintillant (Appendix 1) was added. The samples were counted for [^{3}H]radioactivity by liquid scintillation spectrometry (Beckman 6800). Nonspecific NBMPR binding was defined as the amount of [^{3}H]NBMPR which remained membrane-associated when the assays were conducted in the presence of a high concentration of a specific inhibitor of nucleoside transport such as NBTGR (10 µM), dilazep (200 µM) or dipyridamole (50 µM). Site-specific NBMPR binding was obtained by subtracting nonspecific binding from total binding.

Determination of [^{3}H]NBMPR Binding Constants

Cardiac membranes were incubated for 20 min with a range of concentrations of [^{3}H]NBMPR (0.02, 0.05, 0.1, 0.15, 0.2, 0.25, 0.4, 0.5, 0.75, 1.0, 2.0, 3.0 nM). Total, nonspecific and specific binding was determined for each concentration of [^{3}H]NBMPR. The dissociation constant (Kd) and the maximum binding site density (Bmax) for [^{3}H]NBMPR binding were determined by linear regression analysis of Scatchard plots (bound/free vs bound). Free ligand concentration was calculated by subtracting total bound [^{3}H]NBMPR from the initial ligand concentration.

Effect of Collagenase Treatment on [^{3}H]NBMPR Binding

[^{3}H]NBMPR binding constants were determined for both rat and guinea pig cardiac membranes in the presence and absence of collagenase. This procedure was designed to simulate the conditions under which cardiac myocytes were prepared. Membrane suspensions were divided into two 20 ml aliquots. Sigma type IA collagenase (150 u/ml), and Sigma type V collagenase (300 u/ml) were added to one of the membrane suspensions, and incubated for 40 min at 37°C. The control suspension was also incubated for 40 min at 37°C. Membrane suspensions were then centrifuged at 20,000 g for 20 min at 4°C and supernatants were removed. Treated membrane pellets were resuspended in 20 ml of disruption medium (Appendix 1), and control membrane pellets were resuspended in 20 ml of 50 mM Tris-HCl. Following 20 min incubation at 37°C, both treated and control membrane suspensions were recentrifuged (as above) and washed twice with 50 mM Tris-HCl , and finally resuspended in 20 ml of 50 mM Tris-HCl buffer. [^{3}H]NBMPR binding assays were then conducted to determine the [^{3}H]NBMPR binding constants for both treated and control cardiac membrane suspensions, as previously described.

C. [^{3}H]NBMPR Binding Assays with Myocytes

General Methodology

Equilibrium binding assays were initiated with the addition of myocyte suspensions (0.4 ml containing 30,000-60,000 cells) to mixtures containing appropriate concentrations of [^{3}H]NBMPR in the absence or presence of a specific inhibitor of nucleoside transport. Assays were conducted in a total volume of 1 ml in polypropylene microcentrifuge tubes (1.5 ml) at 22°C. Tissue was incubated for 40 min to obtain equilibrium binding of [^{3}H]NBMPR. At this point, the reaction was stopped by centrifugation (Eppendorf 5412 microcentrifuge for 15 s) to separate the free ligand from cell-associated [^{3}H]NBMPR. Cell pellets were washed once with 1 ml of additional ice-cold assay medium. The myocyte pellets were digested with 0.25 ml 0.5 N KOH overnight. Following neutralization with 0.1 ml 1.25 N HCl, a 0.2 ml aliquot was transfered to a mini-scintillation (5 ml) vial and 5 ml of tritisol scintillant (Appendix 1) was added. This was counted for [^{3}H]radioactivity by liquid scintillation spectrometry (Beckman 6800). Nonspecific NBMPR binding was defined as the amount of [^{3}H]NBMPR which remained cell associated when the assays were conducted in the presence of a high concentration of a specific inhibitor of nucleoside transport such as nitrobenzylthioguanosine (NBTGR, 10 μM) or dilazep (100 μM). Specific NBMPR binding was calculated as the difference between total binding and nonspecific binding. All drugs and cell suspensions were prepared in

modified Dulbecco's phosphate buffered saline (Appendix 1).

Determination of [^{3}H]NBMPR Binding Constants

The dissociation constant (Kd) and the maximum binding site density (Bmax) for [^{3}H]NBMPR binding were determined by linear regression analysis of Scatchard plots (bound/free vs bound). Cardiac myocytes were incubated for 40 min with varying concentrations of [^{3}H]NBMPR (0.02, 0.05, 0.1, 0.15, 0.2, 0.25, 0.4, 0.5, 0.75, 1.0, 2.0, 3.0 nM). Free [^{3}H]NBMPR concentration was calculated by subtracting total bound from the initial ligand concentration.

[^{3}H]NBMPR Binding in the Presence of Adenosine Deaminase

[^{3}H]NBMPR binding constants were determined for both rat and guinea pig myocytes in the presence and absence of adenosine deaminase. Myocyte suspensions were divided into two 20 ml aliquots. Five units of adenosine deaminase was added to one and incubated for 15 min at room temperature. One unit of adenosine deaminase deaminates 10 μmoles of adenosine to inosine per min at pH 7.5, at 25°C. [^{3}H]NBMPR binding assays were then conducted with both treated and control myocyte suspensions, as previously described.

D. Inhibition of [^{3}H]NBMPR Binding to Myocytes

Determination of Inhibition Constants

The binding of a range of [^{3}H]NBMPR concentrations (0.1, 0.12, 0.17, 0.25, 0.5, 1.0 nM) was determined in the presence of two or three concentrations of inhibitor. Inhibition constants (Ki values) were determined for a variety of different agents including dipyridamole, dilazep, diazepam, adenosine and 2-chloroadenosine. The inhibition of the site-specific component of [^{3}H]NBMPR binding was evaluated by mass law analysis using a double reciprocal plot (1/bound vs 1/free). The free ligand concentration was calculated by subtracting total bound from the initial ligand concentration.

Effect of Deoxycoformycin

Inhibition constants (Ki values) were determined for adenosine-mediated inhibition of [^{3}H]NBMPR binding (as previously described) in the presence or absence of the adenosine deaminase inhibitor 2'-deoxycoformycin (100 nM). Three concentration of adenosine were used for both rat (30, 50 and 100 μM) and guinea pig (300 , 800 and 1000 μM) myocyte preparations.

E. [^{3}H]Nucleoside Influx Assays with Cardiac Myocytes

General Methodology

All assays of [^{3}H]nucleoside influx into rat or guinea pig cardiac myocytes were conducted in polypropylene

microcentrifuge tubes (1.5 ml) at 22°C. Uptake intervals were initiated by the addition of 50 µl of an appropriate initial concentration of permeant to myocyte suspensions (200,000 to 500,000 cells/450 µl assay), with subsequent rapid mixing. Uptake intervals were terminated by an inhibitor-stop method by addition of an ice-cold solution (1 ml) containing dilazep (200 µM), NBMPR (40 µM) and 1 mM unlabelled substrate (either adenosine or 2-chloroadenosine) to assay mixtures. Myocytes were pelleted by centrifugation (15 s in a Eppendorf 5412 microcentrifuge) and washed once with additional ice-cold inhibitor solution. Tips of the microcentrifuge tubes were cut off and placed in polypropylene scintillation mini vials (5 ml). The cell pellets were digested overnight with NCS tissue solubilizer (0.3 ml). The following day, glacial acetic acid (9 µl) was added to the vials to neutralize the tissue digests. Toluene scintillant (5 ml; Appendix 1) was added to the mini vials and the contents were counted for [^{3}H]radioactivity by liquid scintillation spectrometry (Beckman 6800). Non-mediated nucleoside influx was defined as the influx of permeant when the assays were conducted in the presence of a high concentration of a nucleoside transport inhibitor such as NBMPR (20 µM) or dilazep (100 µM). Mediated (inhibitable) nucleoside influx was defined as the difference between total influx and non-mediated influx. All drugs and myocyte suspensions were prepared in modified Dulbecco's phosphate buffered saline (Appendix 1).

Time Courses of [^{3}H]Adenosine Uptake

Myocytes were incubated with a fixed concentration of [^{3}H]adenosine over a range of time intervals between zero and 10 min. Time courses of [^{3}H]adenosine uptake were undertaken for 1, 10, 30, 50, 75 and 100 μM concentrations with rat myocytes, and 1, 30, 50, 100, 150 and 300 μM concentrations with guinea pig myocytes. Influx at time zero was determined by the addition of permeant to assays containing high concentration of nucleoside transport inhibitor (such as 20 μM NBMPR or 100 μM dilazep), followed immediately with the addition of stopping solution.

Determination of [^{3}H]Adenosine Transport Kinetic Constants

Rates of zero-trans influx of [^{3}H]adenosine were determined by incubating myocytes in graded concentrations of [^{3}H]adenosine (1 μM to 400 μM final concentration). Incubation intervals were determined from the time courses of [^{3}H]adenosine uptake, and were chosen in order that initial rates of permeant flux were measured. In addition, at time intervals used (15 s), the intracellular concentration of [^{3}H]adenosine did not exceed 20% of the corresponding extracellular concentration. The Michaelis constant (Km) and the maximum velocity (Vmax) of the transport process were derived from linear regression analysis of v/s vs v plots.

F. Inhibition of [^{3}H]Adenosine Transport

Determination of Inhibition Constants

Cardiac myocytes were incubated with a fixed concentration of [^{3}H]adenosine in the presence of varying concentrations of inhibitor. Concentration dependence curves were constructed in this manner for NBMPR (0.1 to 10 nM), dipyridamole (100 nM to 300 μM for rat and 3 nM to 30 μM for guinea pig), dilazep (10 nM to 100 μM for rat and 0.3 nM to 10 μM to guinea pig) and diazepam (3 μM to 300 μM). The concentrations of [^{3}H]adenosine used were 30 μM for rat myocytes and 100 μM for guinea pig myocytes. IC_{50} values were defined as the concentration of inhibitor necessary to inhibit 50 % of the mediated zero-trans influx of [^{3}H]adenosine. Inhibition constants (Ki values) were calculated from the equation $Ki = IC_{50} / (1 + [S])/Km)$ (Marangos *et al.* 1984).

G. [^{3}H]2-Chloroadenosine Influx Assays with Guinea Pig Myocytes

Time-Courses of 2-Chloroadenosine Uptake

Guinea pig myocytes were incubated with a fixed concentration of [^{3}H]2-chloroadenosine over a range of time intervals between zero and 20 min. Time courses were undertaken for 1 and 100 μM 2-chloroadenosine. Influx at time zero was determined by the addition of permeant to

assays containing a high concentration of nucleoside transport inhibitor (such as 20 μM NBMPR or 100 μM dilazep), followed immediately by the addition of stopping solution.

Determination of [^{3}H]2-Chloroadenosine Transport Kinetic Constants

Rates of zero-trans influx of [^{3}H]2-chloroadenosine were determined by incubating guinea pig myocytes in graded concentrations of [^{3}H]2-chloroadenosine (1 to 200 μM final concentration). Incubation intervals were determined from the time courses of [^{3}H]2-chloroadenosine uptake and were chosen in order that initial rates of permeant fluxes were measured. Incubation intervals of 15 s were used for concentrations equal to or less than 30 μM, and 10 s for concentrations exceeding 30 μM. The Michaelis constant (Km) and the maximum velocity (Vmax) of the transport process were derived from linear regression analysis of v/s vs v plots.

H. Inhibition of [^{3}H]2-Chloroadenosine Transport

Determination of Inhibition Constants

Guinea pig cardiac myocytes were incubated with a fixed concentration of [^{3}H]2-chloroadenosine (35 μM) in the presence of varying concentrations of inhibitors. Concentration dependence curves were conducted in this manner for NBMPR (0.1 to 10 nM) and dipyridamole (1 nM to 30

μM). Time intervals of permeant incubation were chosen to ensure that initial rates of permeant influx were measured. An incubation intervals of 15 s was chosen for all assays of the inhibition of 35 μM [^{3}H]2-chloroadenosine transport. IC_{50} values were defined as the concentration of inhibitor necessary to inhibit by 50 % the mediated zero-trans influx of [^{3}H]2-chloroadenosine. Inhibition constants (Ki values) were calculated from the equation $Ki = IC_{50} / (1 + [S]/Km)$.

I. Data Analysis

The data presented represents mean values of between 3 and 7 observations, as indicated. Kd and Km values are geometric means (with 95% confidence limits), and all other values represent arithmetic means (±S.E.). Significance levels were determined by Student's *t*-test for paired or unpaired data, and differences were judged to be significant when $p < 0.05$.

J. Radiochemicals and Drugs

[G-^{3}H]Nitrobenzylthioinosine (specific activity 37 Ci/mmol), [^{3}H]adenosine (specific activity 17.8 Ci/mmol) and [^{3}H]2-chloroadenosine (specific activity 9 Ci/mmol) were obtained from Moravek Biochemicals, California. Unlabelled adenosine, 2-chloroadenosine, dipyridamole, adenosine deaminase, collagenase IA and collagenase V were purchased from Sigma Chemical Co. Dilazep and diazepam were donated by Hoffman-La Roche. Nitrobenzylthioinosine and

nitrobenzylthioguanosine were generously provided by Dr. A.R.P. Paterson, Cancer Research Unit (McEachern Laboratory), University of Alberta. N.C.S. Tissue Solubilizer was purchased from Amersham Corporation. Deoxycoformycin was generously donated by the Natural Products Branch, Division of Cancer Treatment, National Cancer Institute.

III. RESULTS

A. [^{3}H]NBMPR Binding with Cardiac Membranes

Determination of [^{3}H]NBMPR Binding Constants

The "total" amount of [^{3}H]NBMPR that became associated at equilibrium with P_2 membrane preparations from guinea pig and rat ventricles (Fig 1) consisted of two components, one which was proportional to the "free" [^{3}H]NBMPR concentration (nonspecific binding), whereas, the other was site-specific (inhibited by nucleoside transport inhibitors) and saturable. The nonspecific component was approximately 5 % and 15 % of total [^{3}H]NBMPR binding at Kd concentrations in guinea pig and rat ventricular membranes, respectively. Binding constants, derived from mass law analysis of site-specific [^{3}H]NBMPR binding to guinea pig and rat ventricular membranes (Table 1), demonstrated that [^{3}H]NBMPR bound with high affinity to a single class of sites in both guinea pig and rat membranes. [^{3}H]NBMPR possessed slightly higher affinity for these sites in rat membranes (Kd = 0.05 (0.02 - 0.09)nM) as compared to guinea pig membranes (Kd = 0.75 (0.36 - 1.62) nM). Guinea pig possessed a much larger NBMPR site density (Bmax) than rat. The Bmax values for guinea pig and rat ventricular membranes were 1715 ± 96 fmol/mg protein and 178 ± 22 fmol/mg protein, respectively. Hill coefficients (nH; Table 1) did not deviate from unity, indicating that [^{3}H]NBMPR binding to membranes from guinea

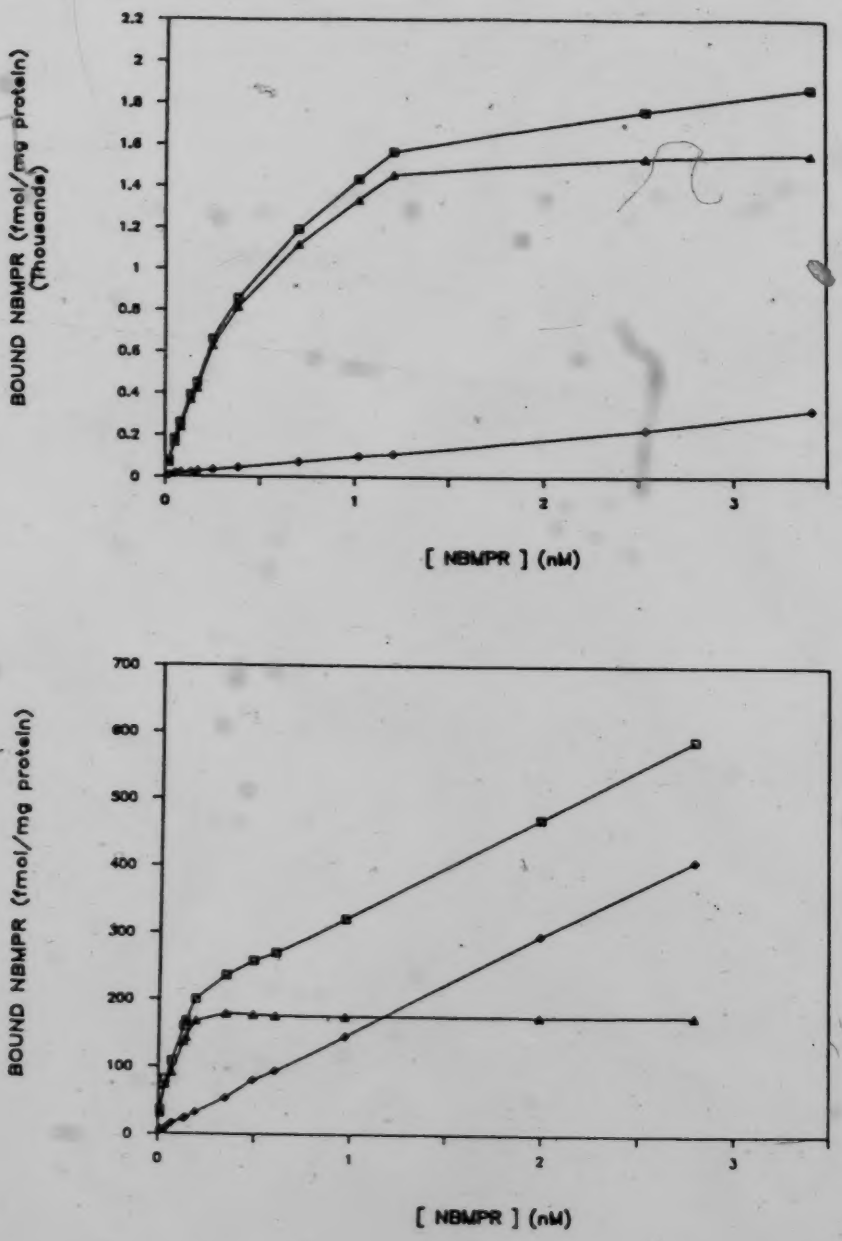


Fig 1. Concentration dependence of [^{3}H]NBMPR binding to guinea pig (upper panel) and rat (lower panel) ventricular membranes. These are representative plots and do not differ from 5 others with guinea pig and 2 others with rat. Total binding (□); nonspecific binding (◇); specific binding (△).

Table 1. [^{3}H]NBMPR binding constants in guinea pig and rat ventricular membranes.

Species (n)	Bmax (fmol/mg protein)	Kd (nM) (95% confidence limits)	nH
Guinea Pig (6)	1715 ± 96	0.75 (0.36-1.62)	1.06 ± 0.01
Rat (3)	178 ± 22	0.05 (0.02-0.09)	1.02 ± 0.01

Values were obtained from the number of experiments in parenthesis (n).

pig and rat ventricles displayed no apparent binding cooperativity.

Effect of Collagenase Treatment

Paired [^{3}H]NBMPR binding assays were conducted with rat and guinea pig membranes that were preincubated in the presence or absence of collagenase (see Methods). In guinea pig membranes, the affinity of [^{3}H]NBMPR was not affected by collagenase treatment ($P > 0.05$; Fig 2); however, collagenase treatment lowered NBMPR site density (Bmax) by approximately 50 % ($P < 0.05$). Collagenase treatment of rat ventricular membranes significantly ($P < 0.05$) lowered [^{3}H]NBMPR affinity compared to paired controls. [^{3}H]NBMPR binding site density in rat membranes was not significantly ($P > 0.05$) altered by collagenase treatment.

B. [^{3}H]NBMPR Binding with Cardiac Myocytes

Determination of [^{3}H]NBMPR Binding Constants

The total amount of [^{3}H]NBMPR that became associated at equilibrium with ventricular myocytes from guinea pig and rat (Fig 3) consisted of two components, nonspecific binding and site-specific binding. Nonspecific binding of [^{3}H]NBMPR was proportional to the "free" [^{3}H]NBMPR concentration which remained in the assay medium. At Kd concentrations this component was approximately 15 % of total binding in guinea pig myocytes and 20 % of total binding in rat myocytes.

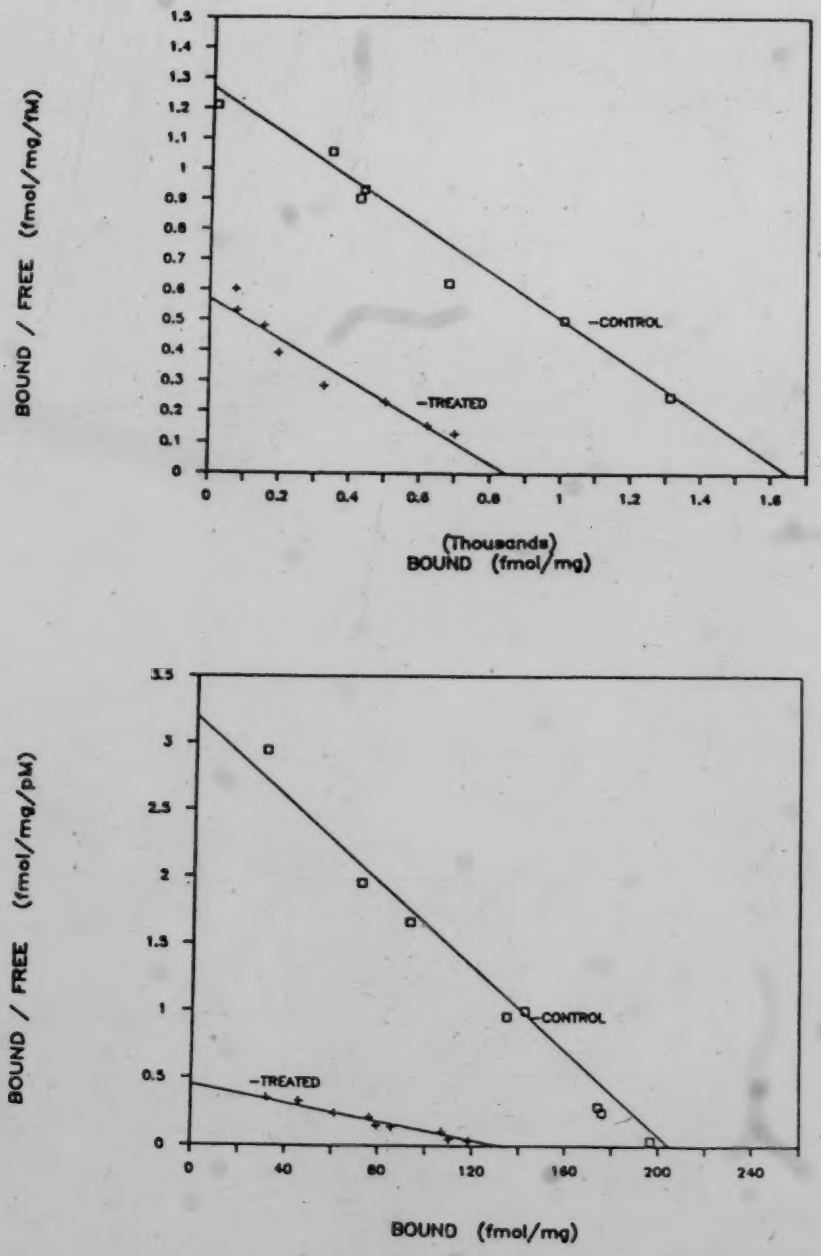


Fig 2. The effect of collagenase treatment on [^{3}H]NBMPR binding to guinea pig (upper panel) and rat (lower panel) ventricular membranes. These are representative plots which do not differ from 5 others with guinea pig and 2 others with rat.

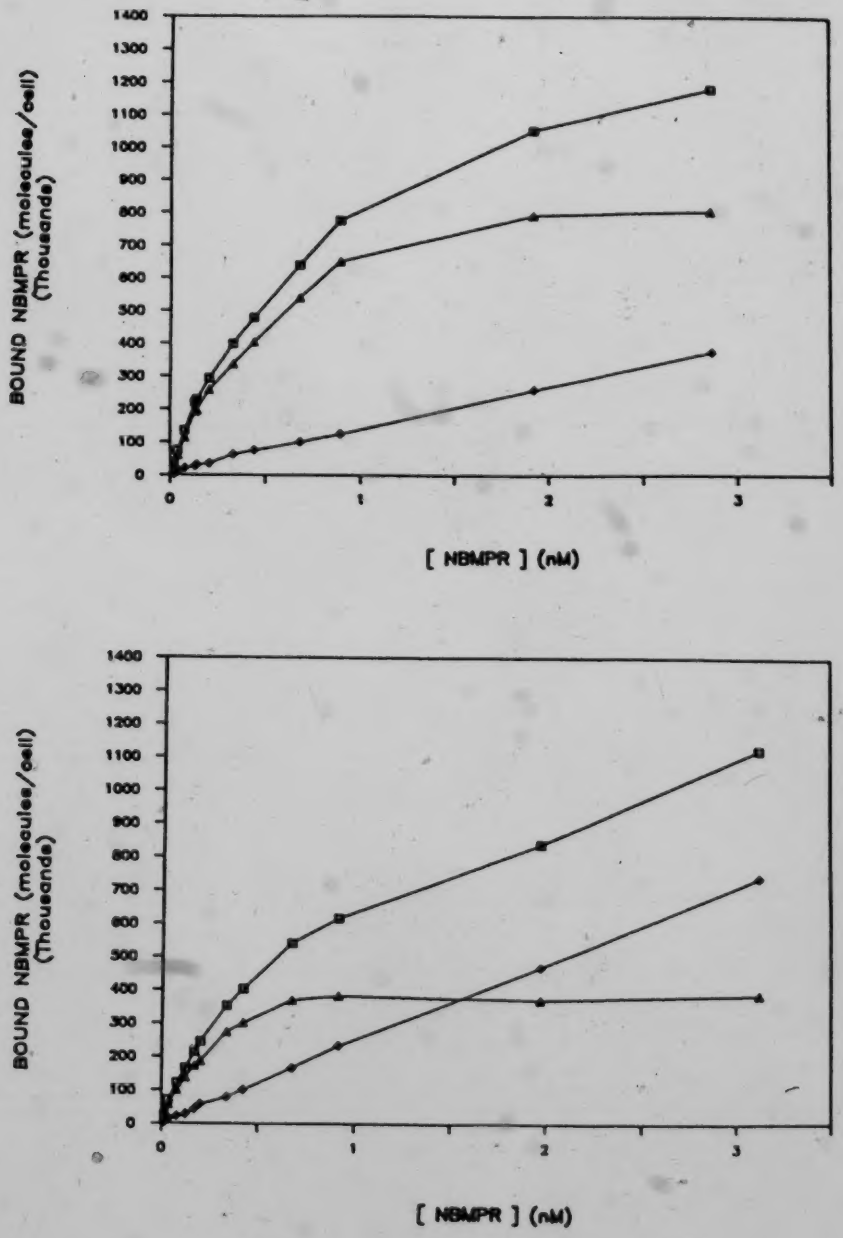


Fig 3. Concentration dependence of [^{3}H]NBMPR binding to guinea pig (upper panel) and rat (lower panel) ventricular myocytes. These are representative plots and do not differ from 5 other experiments with guinea pig and 6 other experiments with rat. Total binding (□); nonspecific binding (◇); specific binding (△).

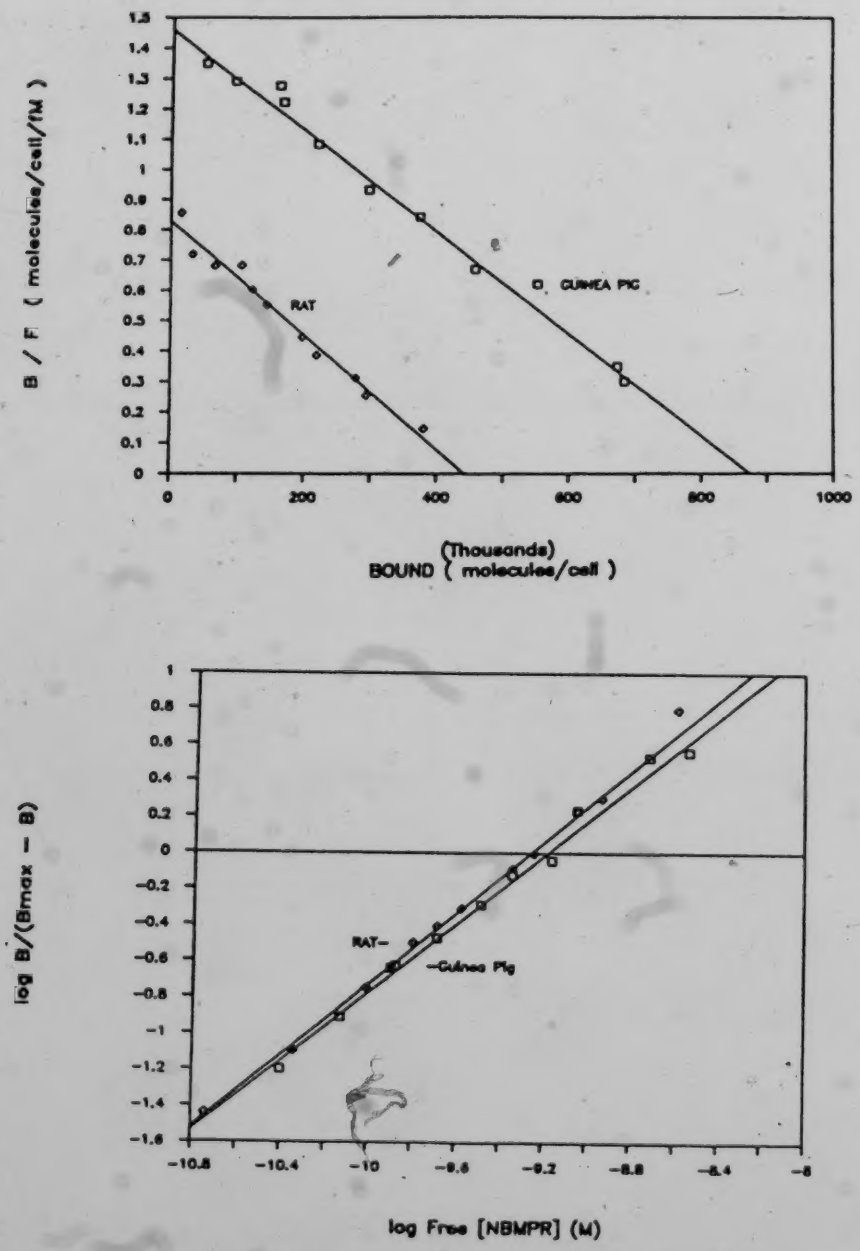


Fig 4. Scatchard (upper panel) and Hill (lower panel) plots of [^{3}H]NBMPR binding to ventricular myocytes. These are representative plots which do not differ from 5 others with guinea pig and 6 others with rat.

Table 2. [^{3}H]NBMPR binding constants in guinea pig and rat ventricular myocytes.

Species (n)	Bmax (molec./cell)	Kd (nM) (95% confidence limits)	nH
Guinea Pig (6)	870 ±36 K	0.76 (0.58-1.00)	1.00 ± 0.02
Rat (7)	437 ±45 K	0.40 (0.29-0.56)	0.99 ± 0.01

Values were calculated from the number of experiment in parenthesis (n). Bmax values are represented in thousands (K).

Binding constants, derived from mass law analysis of site-specific [^{3}H]NBMPR binding to guinea pig and rat ventricular myocytes, demonstrate that [^{3}H]NBMPR bound in a saturable manner to a single class of sites (Fig 4, Table 2). Mean Kd values (95 % confidence intervals) for [^{3}H]NBMPR binding to guinea pig and rat ventricular myocytes were 0.76 (0.58 - 1.00) nM and 0.40 (0.29 - 0.56) nM, respectively (Table 2). Guinea pig myocytes possessed approximately twice as many high affinity NBMPR binding sites per cell as rat myocytes (Fig 4; Table 2). The mean Bmax values (± S.E.) for guinea pig and rat ventricular myocytes were 870,000 ± 36,000 molecules/cell and 437,000 ± 45,000 molecules/cell, respectively. [^{3}H]NBMPR binding to guinea pig and rat myocytes did not display binding cooperativity, as Hill coefficients (nH; Table 2) did not deviate from unity.

Effect of Adenosine Deaminase on [^{3}H]NBMPR Binding Constants

The equilibrium binding of [^{3}H]NBMPR to guinea pig and rat ventricular myocytes was determined in the presence or absence of adenosine deaminase (Fig 5). [^{3}H]NBMPR bound to adenosine deaminase-treated myocytes in a similar manner as to paired controls. Adenosine deaminase treatment did not alter significantly the affinity (Kd values) of [^{3}H]NBMPR for its high affinity sites on guinea pig ($P > 0.05$) and rat myocytes ($P > 0.05$). In addition, significant differences (from paired controls) in NBMPR site density (Bmax) were not

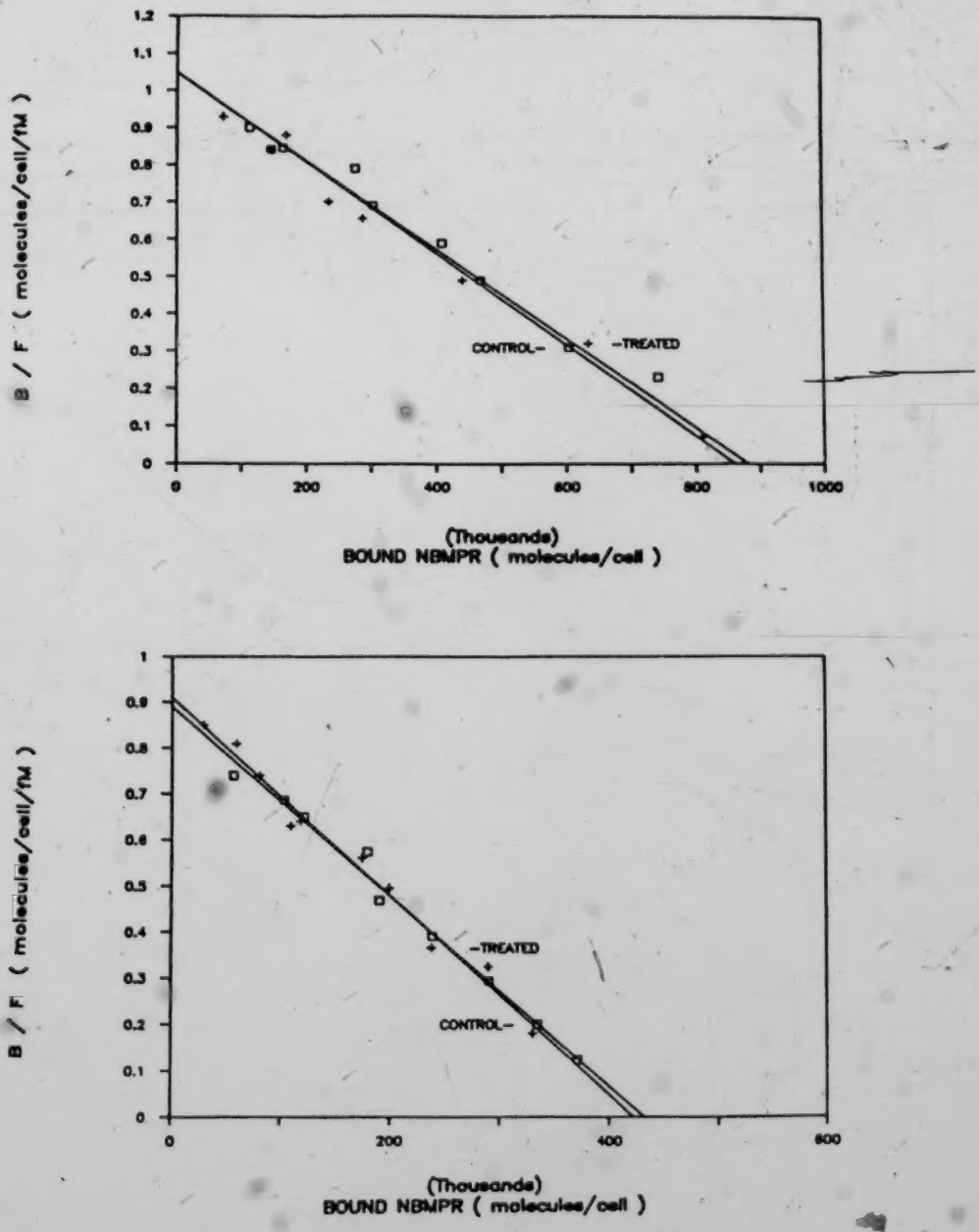


Fig 5. The effect of adenosine deaminase on [^{3}H]NBMPR binding to guinea pig (upper panel) and rat (lower panel) ventricular myocytes. These are representative plots which do not differ from 2 other experiments with both species.

obtained for either guinea pig ($P > 0.05$) or rat myocytes ($P > 0.05$) with adenosine deaminase treatment.

C. Inhibition of [^{3}H]NBMPR Binding to Myocytes

Determination of Inhibition Constants

Competition experiments were performed in order to determine the inhibition constants (Ki values) of a variety of drugs for inhibition of site-specific [^{3}H]NBMPR binding to guinea pig and rat ventricular myocytes (Table 3). The drugs tested (dipyridamole, dilazep, diazepam, adenosine and 2-chloroadenosine) all inhibited [^{3}H]NBMPR binding in an apparently competitive manner, as indicated by double reciprocal plot analysis (Figs 6, 7, 8, 9, and 10). Dipyridamole possessed greater than 20-fold higher affinity for NBMPR sites on guinea pig myocytes (Ki = 75 (44 - 132) nM), as compared to rat myocytes (Ki = 1.7 (1.1 - 2.5) μM). Similarily, dilazep showed a slightly higher affinity for NBMPR binding sites on guinea pig cells (Ki = 6.7 (3.0 - 15.0) nM) than rat (Ki = 34 (21 - 54) nM). The benzodiazepine, diazepam, bound with similar low affinity in both species, with Ki values in guinea pig and rat myocytes of 36 (19 - 70) μM and 43 (13 - 146) μM, respectively. The nucleosides adenosine and 2-chloroadenosine also competitively inhibited [^{3}H]NBMPR binding in both species, however, with differing affinities. 2-Chloroadenosine bound to myocytes from both species with similar affinity; Ki

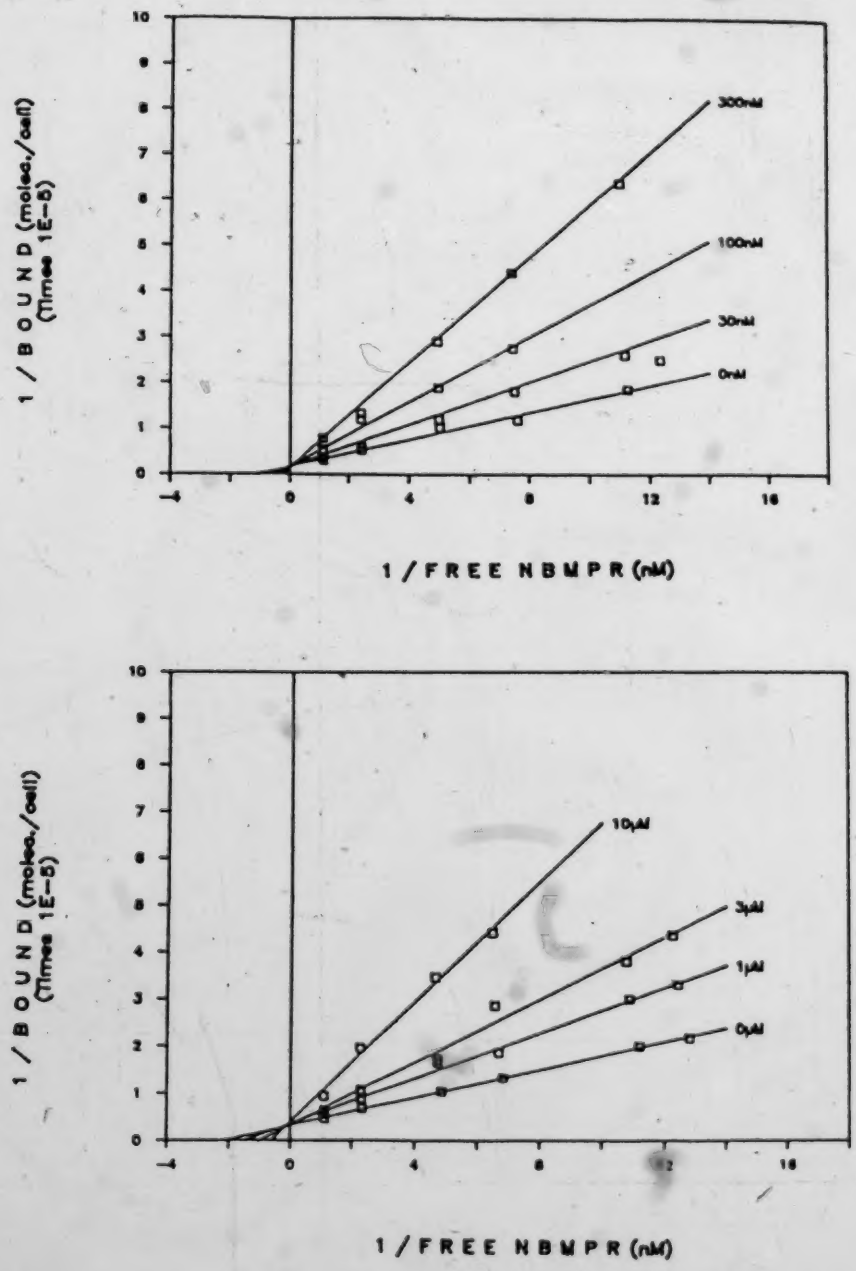
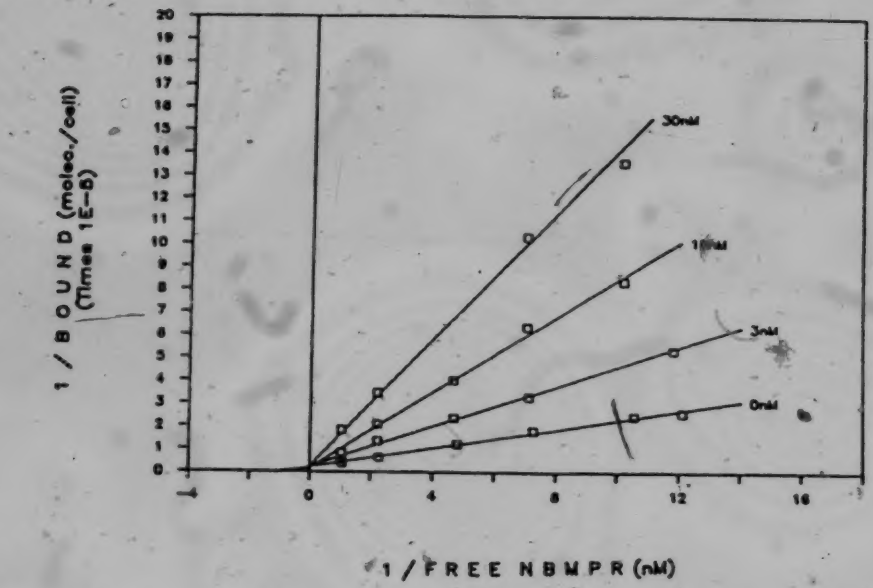


Fig 6. Dipyridamole-mediated inhibition of [^{3}H]NBMPR binding to guinea pig (upper panel) and rat (lower panel) ventricular myocytes. These are representative double reciprocal plots obtained from single experiments performed in duplicate.



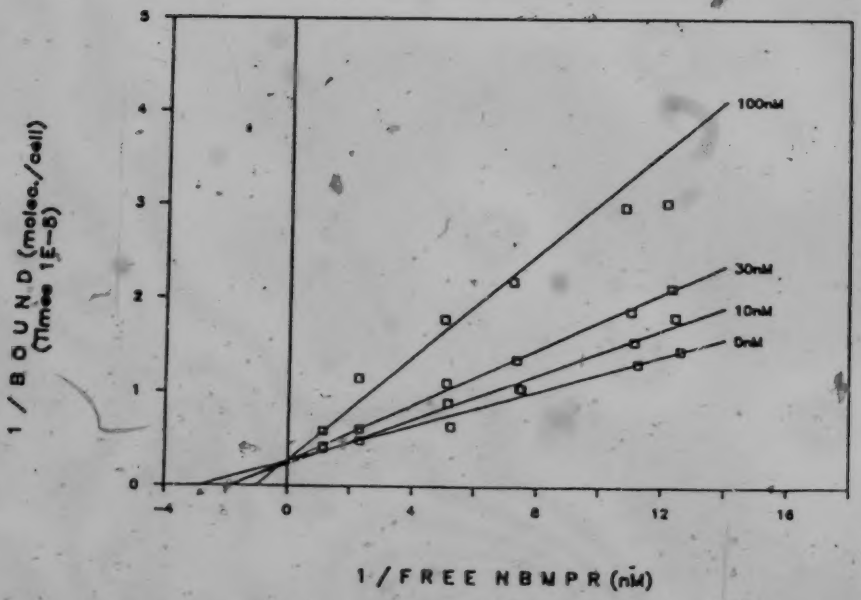


Fig 7. Dilazep-mediated inhibition of [^{3}H]NBMPR binding to guinea pig (upper panel) and rat (lower panel) ventricular myocytes. These are representative double reciprocal plots obtained from single experiments performed in duplicate.

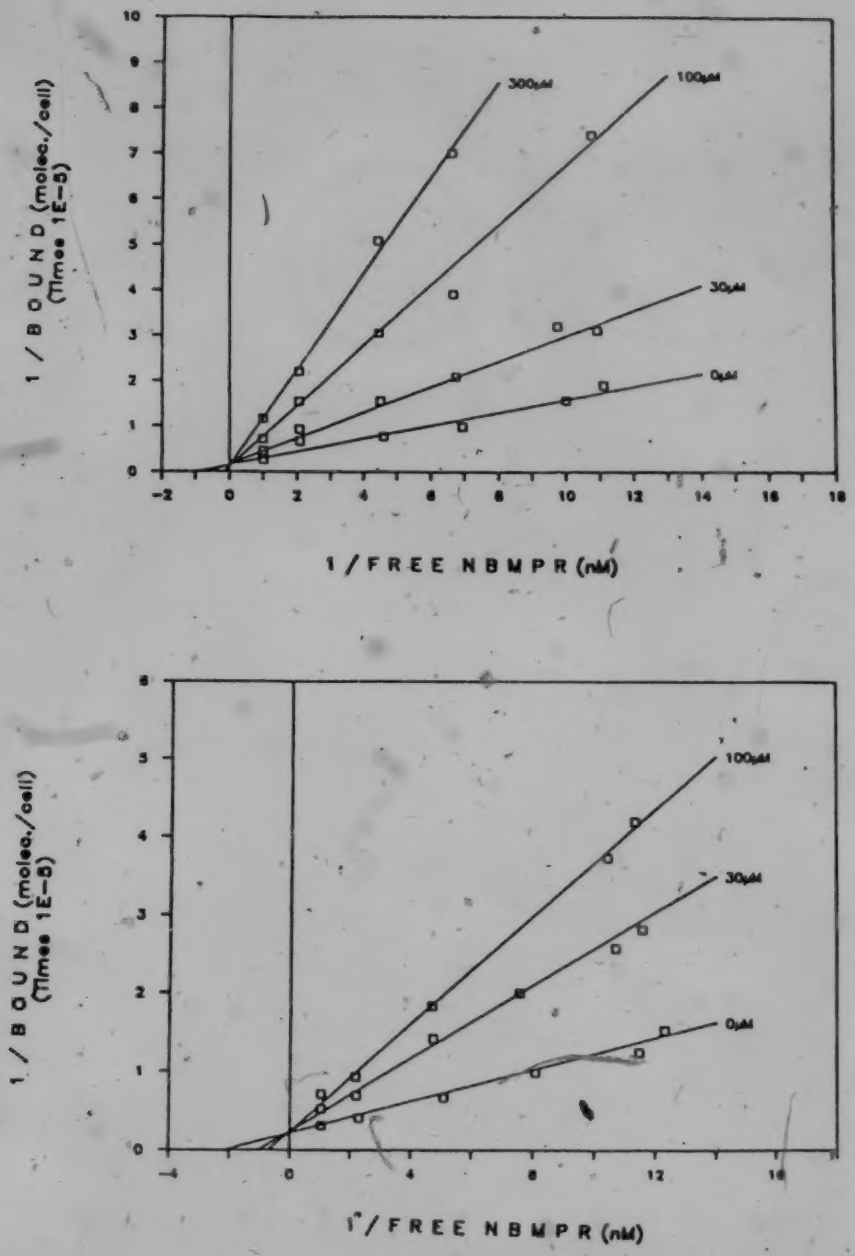
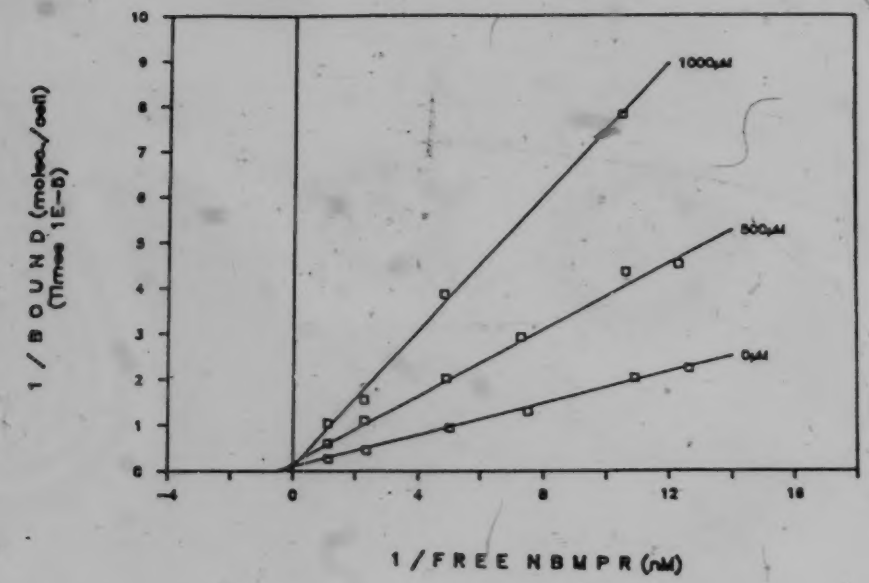


Fig 8. Diazepam-mediated inhibition of [^{3}H]NBMPR binding to guinea pig (upper panel) and rat (lower panel) ventricular myocytes. These are representative double reciprocal plots obtained from single experiments performed in duplicate.



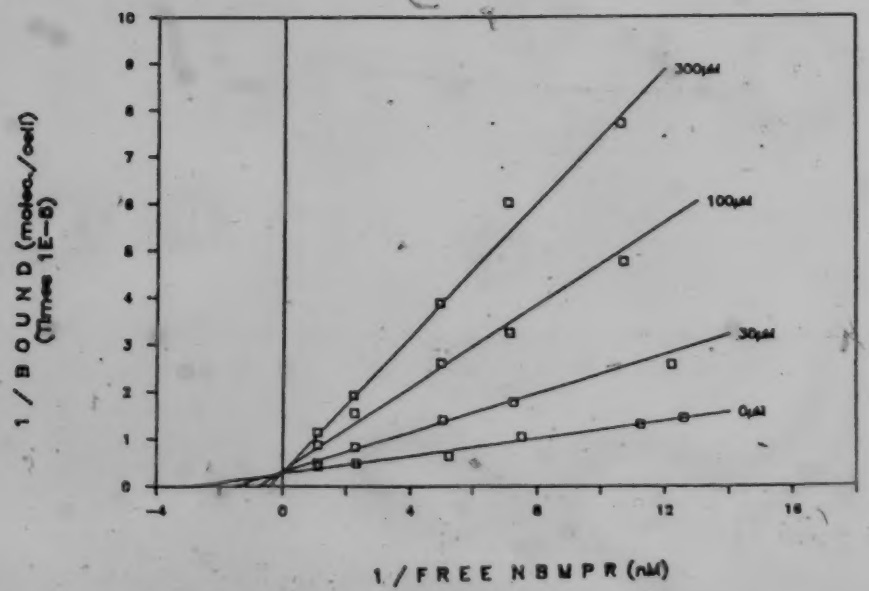
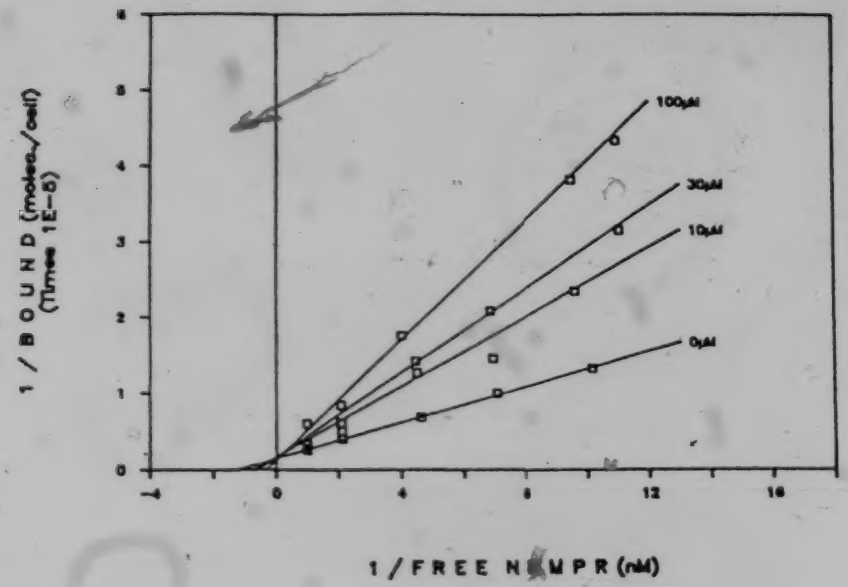


Fig 9. Adenosine-mediated inhibition of [^{3}H]NBMPR binding to guinea pig (upper panel) and rat (lower panel) ventricular myocytes. These are representative double reciprocal plots obtained from single experiments performed in duplicate.



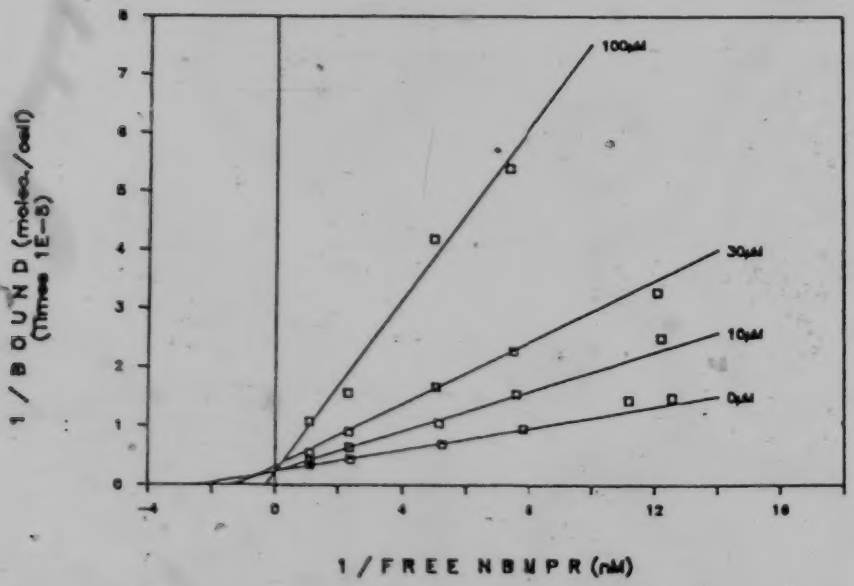


Fig 10. 2-Chloroadenosine-mediated inhibition of [^{3}H]NBMPR binding to guinea pig (upper panel) and rat (lower panel) ventricular myocytes. These are representative double reciprocal plots obtained from single experiments performed in duplicate.

Table 3. Inhibition of site-specific binding of [^{3}H]NBMPR to ventricular myocytes.

	Guinea Pig (n)	Rat (n)
Drug	Ki (nM) (95% confidence limits)	Ki (nM) (95% confidence limits)
Dipyridamole	75 (44 - 132) (6)	1700 (1100-2500) (6)
Dilazep	6.8 (3.0 - 15.0) (6)	34 (21 - 54) (7)
	Ki (μM)	Ki (μM)
Diazepam	36 (19 - 70) (3)	43 (13 - 65) (3)
2-Chloroadenosine	22 (4 - 42) (3)	14 (10 - 19) (3)
Adenosine	318 (284 - 353) (3)	35 (16 - 77) (3)

Inhibition constants were calculated from values obtained from the number of experiments in parenthesis.

values of 22 (4 - 52) μM for guinea pig and 14 (10 - 19) μM for rat. However, adenosine was a more potent inhibitor of site specific [^{3}H]NBMPR binding to rat myocytes (Ki = 35 (16 - 77) μM) than to guinea pig myocytes (Ki = 318 (284 - 353) μM).

Effect of Deoxycoformycin

Adenosine-mediated inhibition of [^{3}H]NBMPR binding was measured in the presence or the absence of the adenosine deaminase inhibitor deoxycoformycin. The inhibition constants (Ki values) for deoxycoformycin-treated myocytes were not significantly different from paired untreated controls in both guinea pig ($P > 0.05$) and rat ($P > 0.05$) preparations.

D. Nucleoside Influx

Time-Courses of Adenosine Uptake

Time-courses of 1 μM adenosine uptake were linear up to 90 s for guinea pig and 2 min for rat myocytes (Fig 11). Time-courses of 100 μM adenosine uptake into guinea pig and rat myocytes were linear up to 30 s and 60 s, respectively (Fig 12). Substrate that became cell-associated in the presence of nucleoside transport inhibitor (most likely representing passive diffusion and equilibration with the extracellular space of the pellet) was linear and proportional to incubation time for intervals up to about

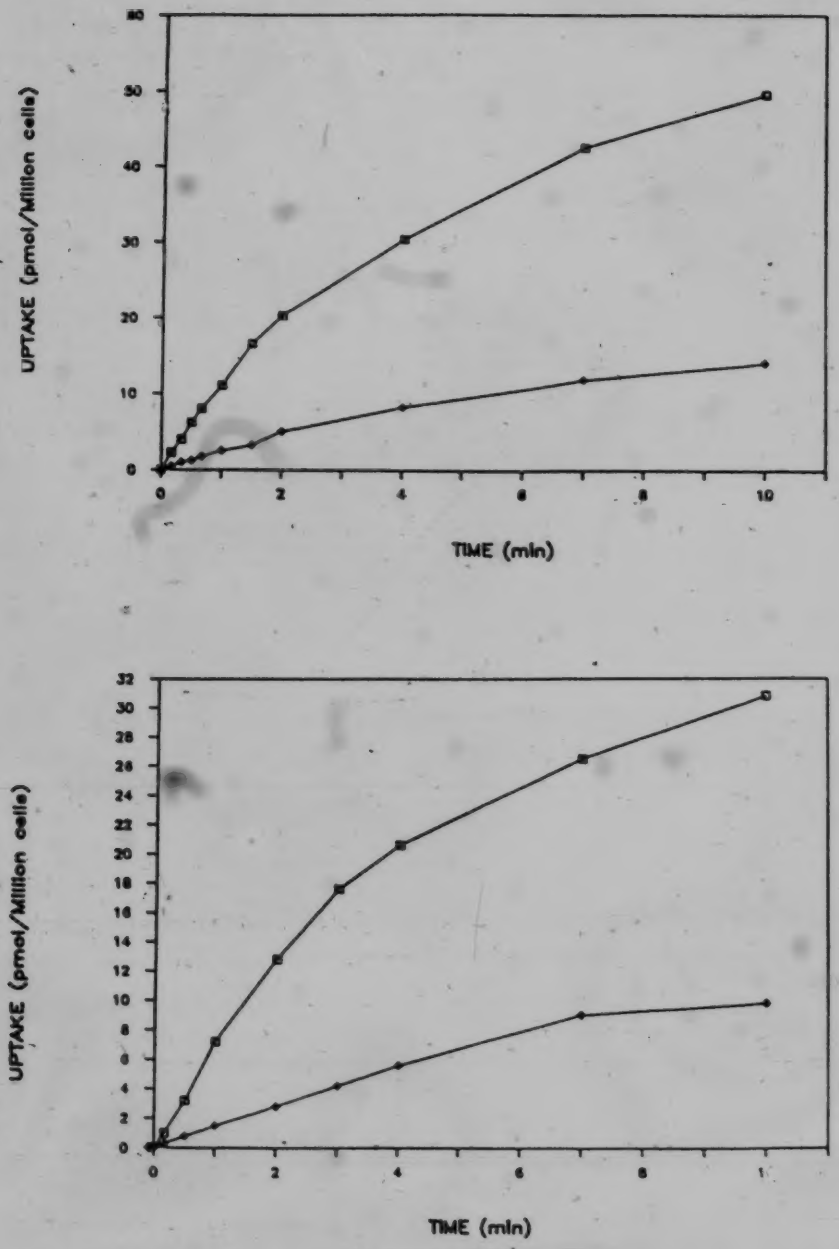


Fig 11. Time-courses of 1 μM adenosine uptake into guinea pig (upper panel) and rat (lower panel) ventricular myocytes. Total uptake (□); uptake in the presence of the nucleoside transport inhibitor, dilazep (100 μM,◆).

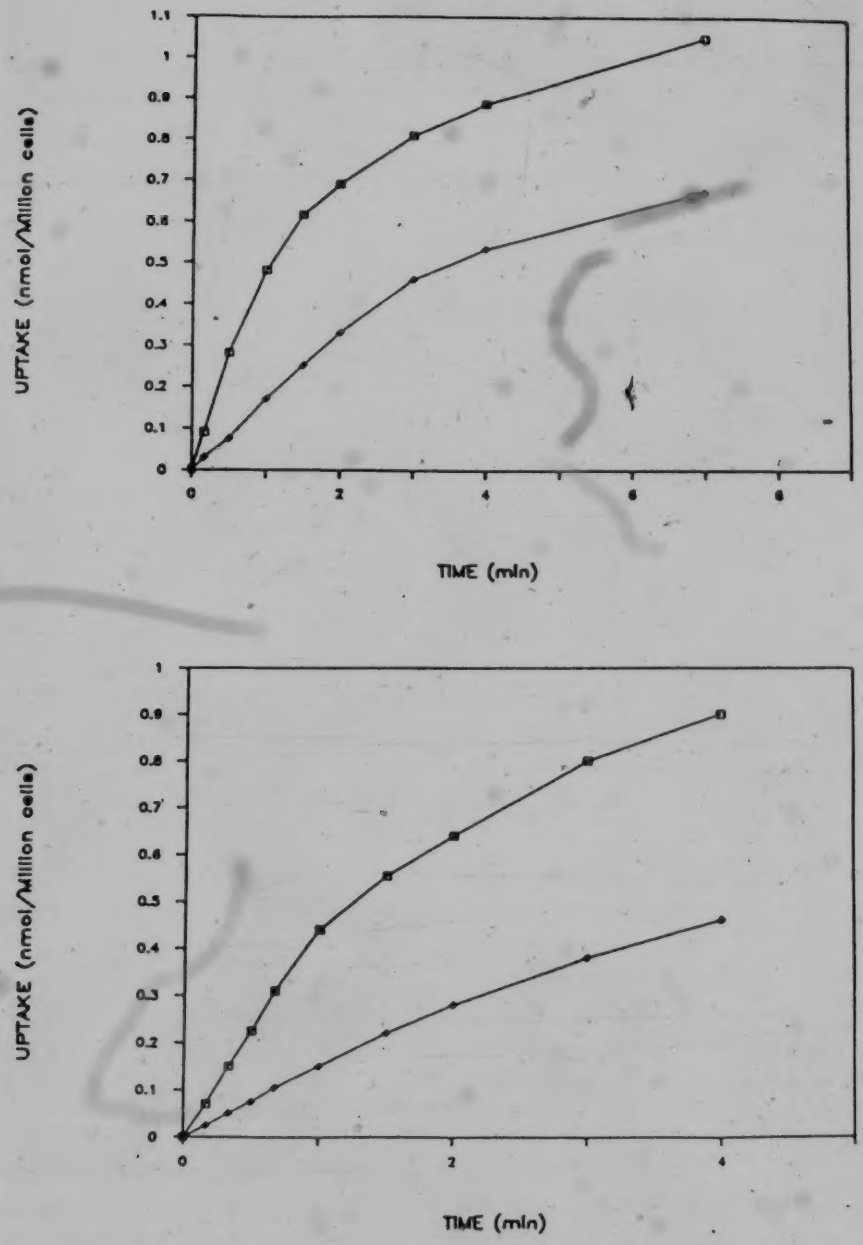


Fig 12. Time-courses of 100 μM adenosine uptake into guinea pig (upper panel) and rat (lower panel) ventricular myocytes. Total uptake (□); uptake in the presence of the nucleoside transport inhibitor, NBMPR (20 μM ♦)

2 min, and declined thereafter. After 4 min of uptake, the radioactivity derived from [^{3}H]adenosine (1 and 100 μM) concentrated approximately 6-fold and 2-fold, respectively, in guinea pig myocytes, and 2-fold for both concentrations in rat myocytes.

Adenosine Transport

Care was taken to ensure that initial rates of nucleoside uptake, representative of transport rates, were measured. The zero-*trans* influx of adenosine into guinea pig and rat ventricular myocytes was found to be saturable. and inhibitable by nucleoside transport inhibitors such as NBMPR and dilazep (Fig. 13; Table 4). The Km (95 % confidence limits) and Vmax (mean ± S.E.) values for adenosine influx into guinea pig myocytes were 146 (101 - 210) μM and 24.2 ± 1.4 pmol/10^{4}cells/s, respectively. Adenosine possessed a lower maximum velocity of transport into rat myocytes (Vmax = 7.1 ± 0.2 pmol/10^{4}cells/s), but possessed a higher affinity for transporters in rat (Km = 50 (38 - 67) μM), as compared to guinea pig.

Time-Courses of 2-Chloroadenosine Uptake

Time-courses of 1 and 100 μM 2-chloroadenosine uptake into guinea pig myocytes were linear for intervals of up to 60 s. The uptake of substrate in the presence of nucleoside transport inhibitor was linear and proportional to incubation time up to about 2 min, and declined thereafter.

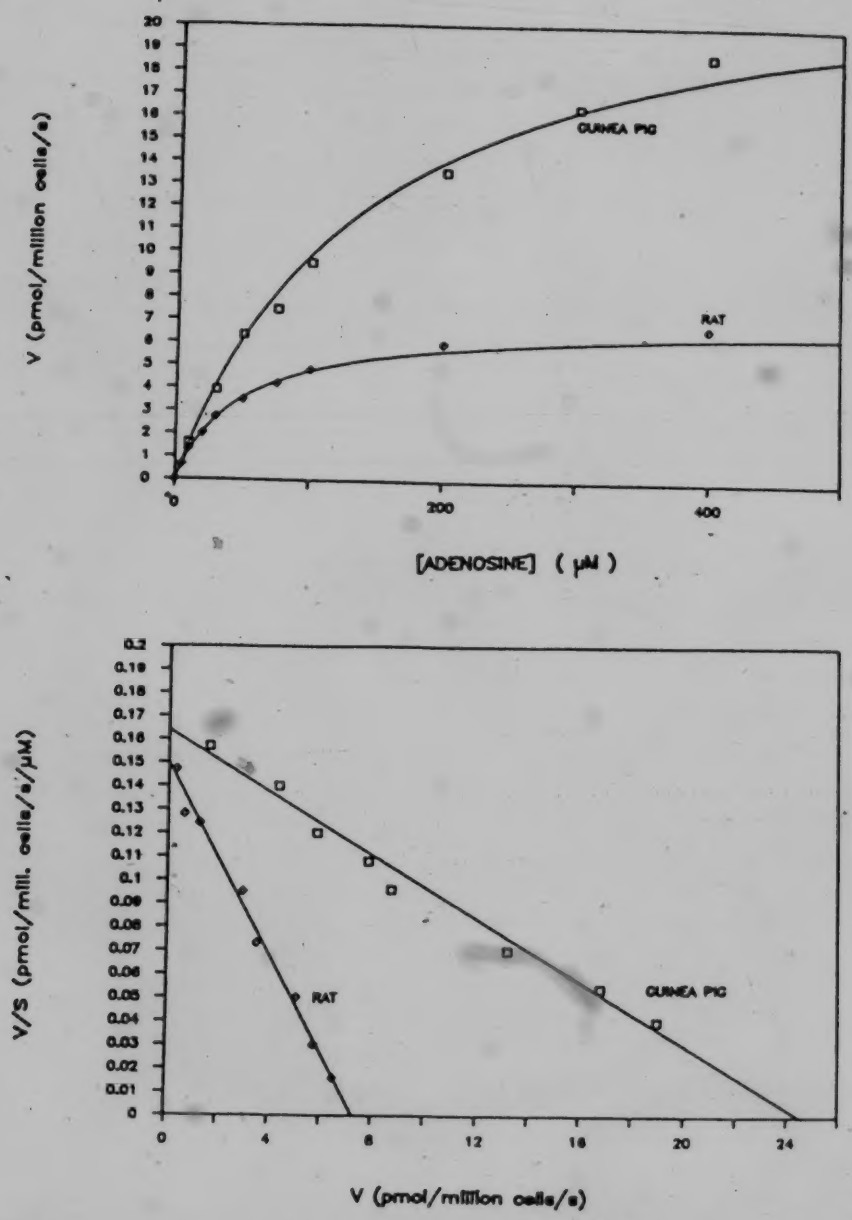


Fig 13. Concentration dependence (upper panel) and V/S vs V (lower panel) plots of adenosine transport into guinea pig and rat ventricular myocytes. The lines in each panel represent computer generated values determined from the average kinetic constants from 3 separate experiments performed in duplicate. The points on each line are the mean values obtained from representative experiments.

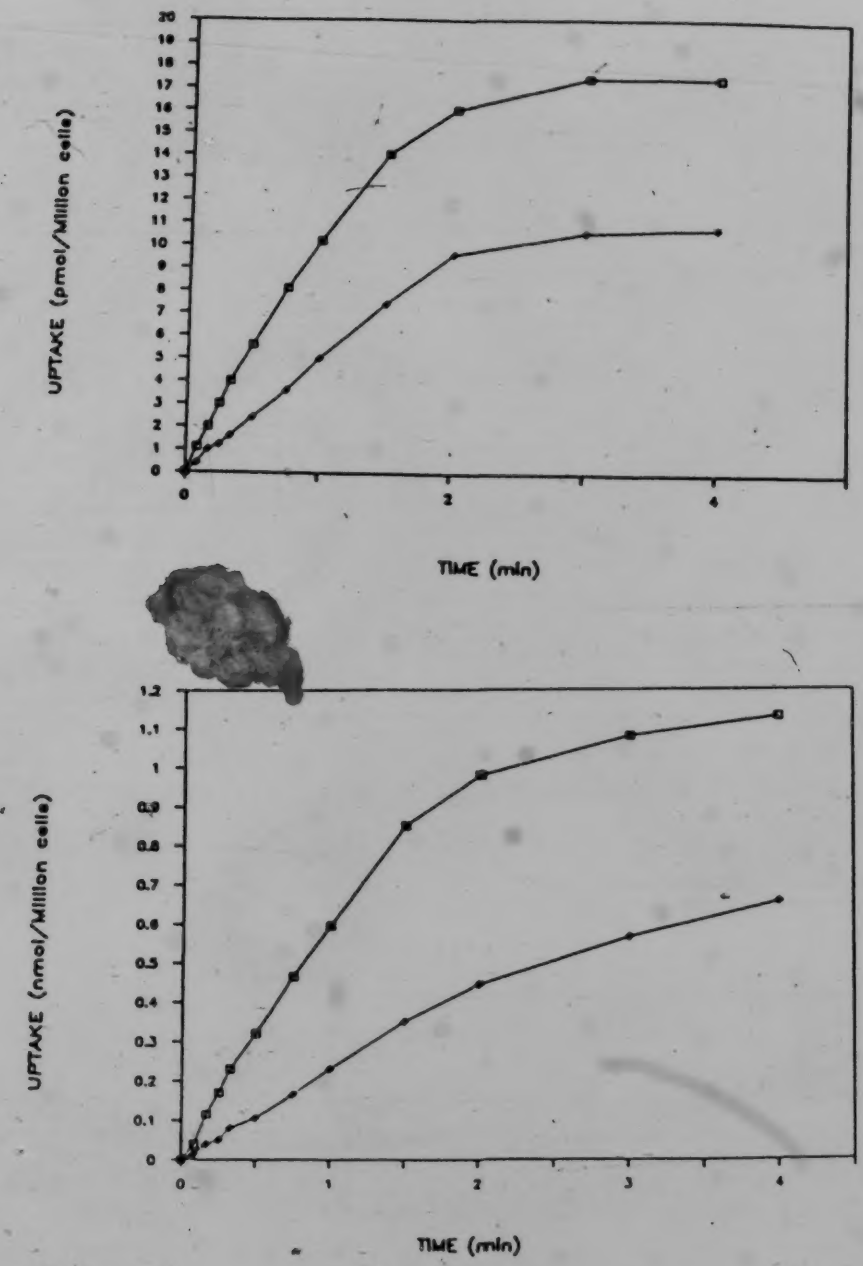


Fig 14. Time-courses of 1 μM (upper panel) and 100 μM (lower panel) 2-chloroadenosine uptake into guinea pig ventricular myocytes. Total uptake (□); uptake in the

Table 4. Nucleoside transport kinetic constants in ventricular myocytes.

	Vmax (pmol/10^6 cells/s)	Km (μM) (95% confidence limits)
Adenosine		
Guinea Pig (3)	24.2 ± 1.4	146 (101 - 210)
Rat (3)	7.1 ± 0.2	50 (38 - 67)
2-Chloroadenosine		
Guinea Pig (3)	11.7 ± 0.1	36 (34 - 39)

The Km and Vmax values were obtained from the number of experiments in parenthesis (n).

The cell-associated radioactivity derived from 100 μM [^{3}H]2-chloroadenosine did not significantly exceed a tissue/medium ratio of unity after 4 min of incubation. However, radioactivity derived from 1 μM [^{3}H]2-chloroadenosine concentrated approximately 1.5-fold after 4 min of incubation.

2-Chloroadenosine Transport into Guinea Pig Myocytes

2-Chloroadenosine was found to be a substrate for the nucleoside transport process in guinea pig myocytes. The zero-*trans* influx of 2-chloroadenosine into guinea pig myocytes was saturable (Fig 15; Table 4) and inhibitable by various known nucleoside transport inhibitors such as NBMPR and dilazep. [^{3}H]2-Chloroadensine had a higher affinity than adenosine for the transport process, with a Km value of 36 (34 - 39) μM. However, 2-chloroadenosine possessed a lower maximum velocity of transport (Vmax = 11.7 ± 0.1 pmol/10^{6}cells/s).

Inhibition of Adenosine Transport

A number of agents including NBMPR, dilazep, dipyridamole and diazepam inhibited the transport of [^{3}H]adenosine into guinea pig and rat myocytes in a concentration dependent manner (Fig 16; Table 6). The inhibitable component of adenosine influx into guinea pig cells (100 μM) and rat cells (30 μM) was approximately 50 %

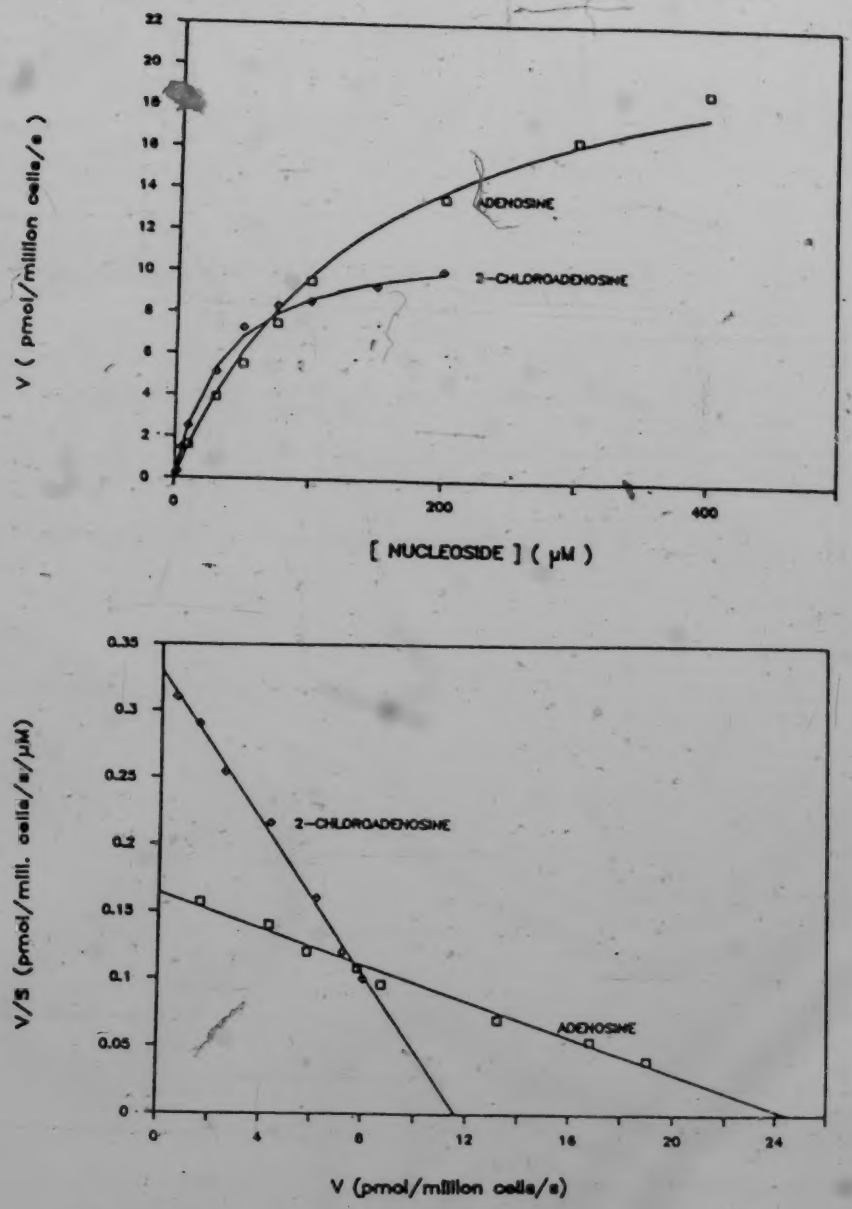


Fig 15. Concentration dependence (upper panel) and V/S vs V (lower panel) plots of 2-chloroadenosine transport into guinea pig ventricular myocytes. The lines in each panel represent computer generated values determined from the average kinetic constants from 3 separate experiments performed in duplicate. The points on each line are the mean values obtained from representative experiments.

pontent inhibitor of adenosine transport into guinea pig and rat myocytes with Ki values of 2.8 (1.0 - 7.6) nM and 2.4 (0.2 - 14.4) nM, respectively. Dipyridamole was a much more potent inhibitor of adenosine transport in guinea pig (Ki = 78 (60 - 101) nM) than in rat (Ki = 3.6 (1.6 - 8.5) μM). Similarily, dilazep was a more potent inhibitor of adenosine transport in guinea pig than rat with Ki values of 8.8 (5.9 - 13.2) nM and 260 (109 - 579) nM, respectively. Finally, the benzodiazepine diazepam inhibited adenosine transport into both species with similar low potency, having Ki values of 27 (11 - 68) μM with guinea pig cells and 52 (20 - 141) μM with rat cells.

Inhibition of 2-Chloroadenosine Transport into Guinea Pig Myocytes

NBMPR and dipyridamole inhibited the transport of [^{3}H]2-chloroadenosine into guinea pig myocytes in a concentration-dependent manner (Fig 18,Table 5). The inhibitable component of 2-chloroadenosine (35μM) influx was approximately 55% of total influx. NBMPR was a potent inhibitor of 2-chloroadenosine transport into guinea pig myocytes, with a Ki value of 3.4 (2.2 - 6.9) nM. Dipyridamole inhibited 2-chloroadenosine transport into guinea pig myocytes with an inhibition constant of 70 (58 - 94) nM.

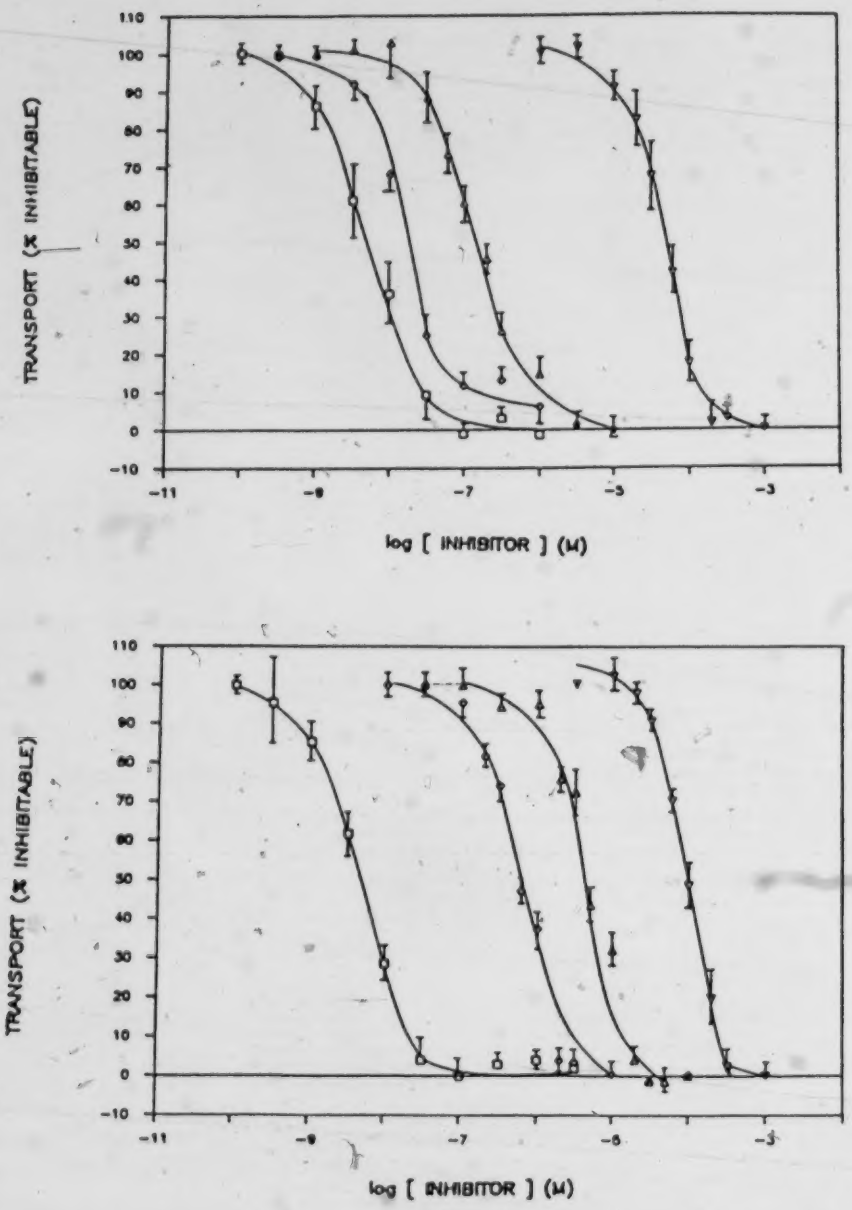


Fig 16. Drug-induced inhibition of 100 μM adenosine transport into guinea pig myocytes (upper panel) and 30 μM adenosine transport into rat myocytes (lower panel). IC_{50} values were obtained from 3 separate experiments performed in quadruplicate. NBMPR (□); dipyridamole (△); dilazep (◇); diazepam (▽).

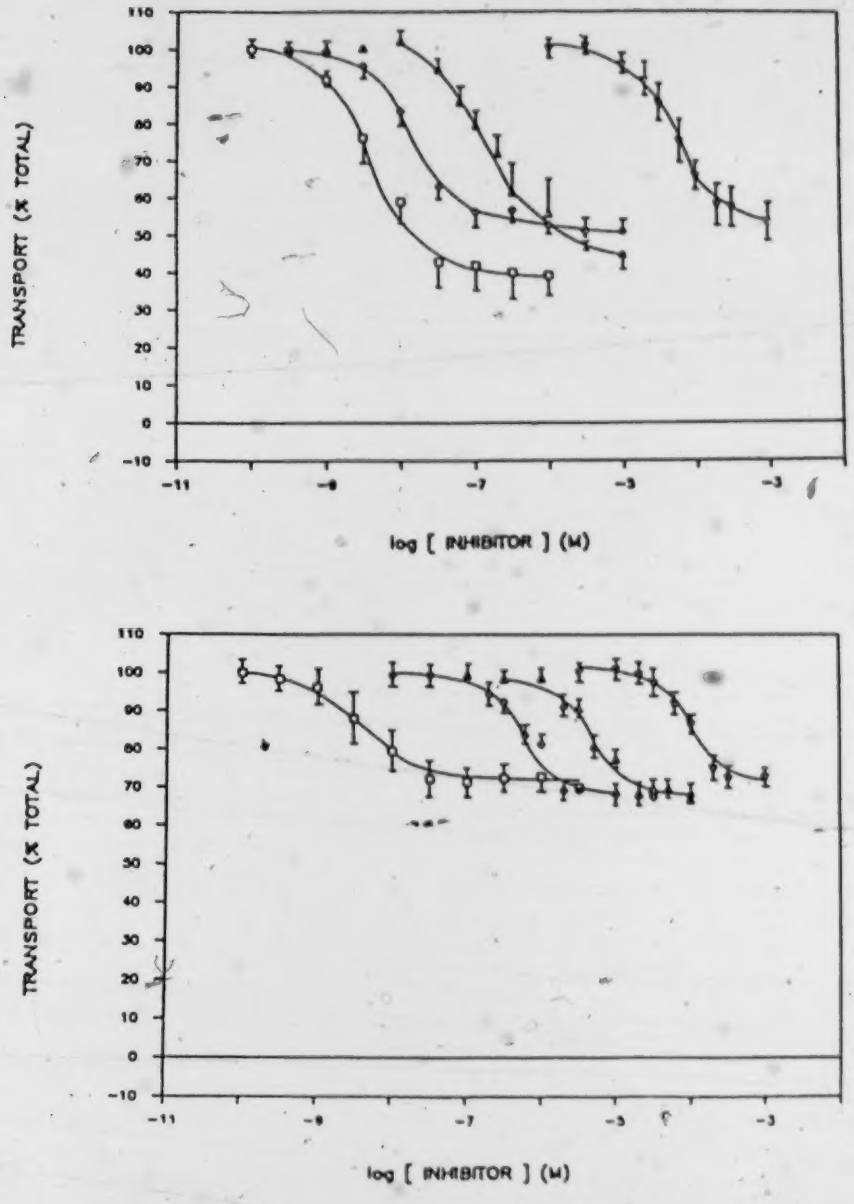


Fig 17. Inhibitable component of 100 μM adenosine transport into guinea pig myocytes (upper panel) and 30 μM adenosine transport into rat myocytes (lower panel). IC_{50} values were obtained from 3 separate experiments performed in quadruplicate. NBMPR (□); dipyridamole (△); dilazep (◇); diazepam (▽). This figure is from the same data as in figure 16.

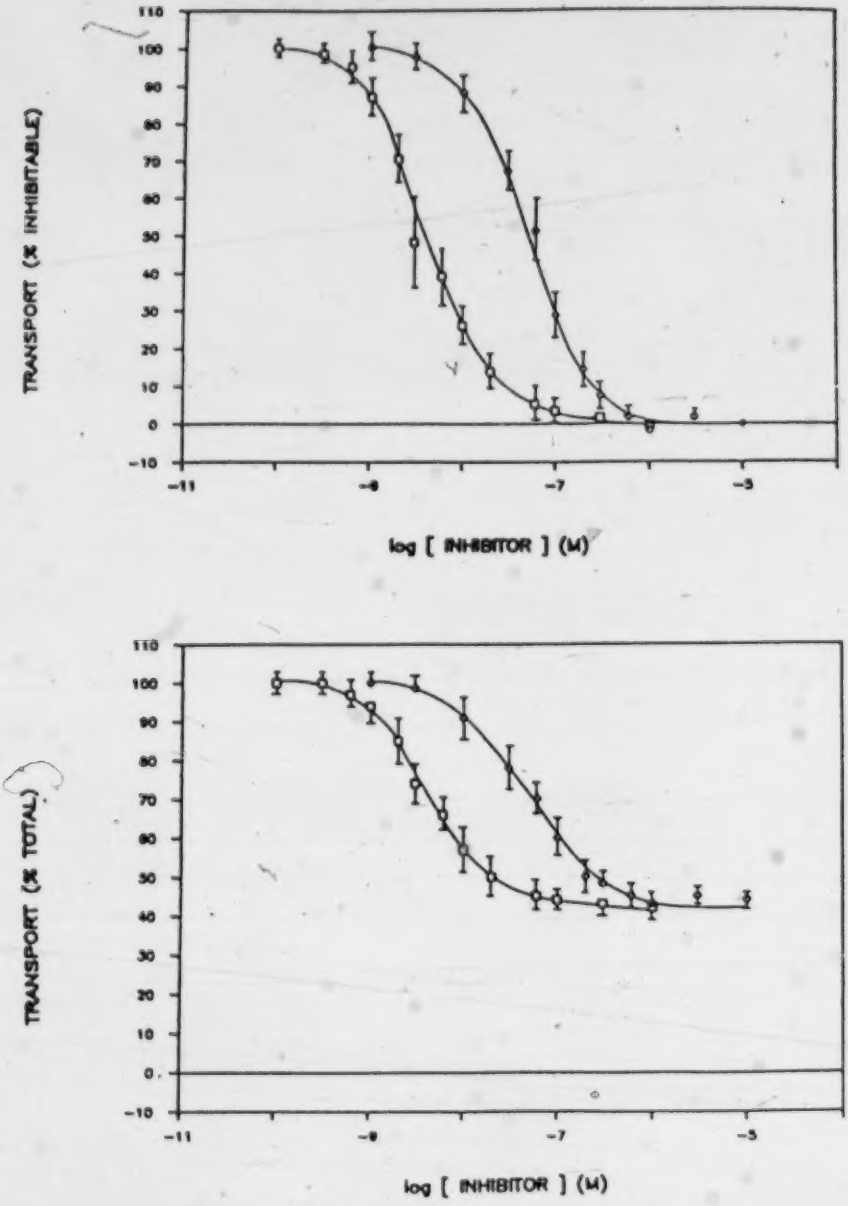


Fig 18. Inhibition of 2-chloroadenosine transport into guinea pig myocytes. Drug-induced inhibition of 35 μM 2-chloroadenosine transport (upper panel). Inhibitable component of 35 μM 2-chloroadenosine transport (lower panel). NBMPR (□); dipyridamole (◇). Both panels were prepared using the same data.

Table 5. Inhibition of adenosine and 2-chloroadenosine transport into ventricular myocytes.

	Guinea pig	Rat
ADENOSINE	Ki (nM)	Ki (nM)
NBMPR	2.8 (1.0 - 7.6)	2.4 (0.2 - 14.4)
Dipyridamole	78 (60 - 101)	3600 (1600 - 8500)
Dilazep	8.8 (5.9 - 13.2)	260 (109 - 579)
	Ki (μM)	Ki (μM)
Diazepam	27 (11 - 68)	52 (20 - 141)
2-CHLOROADENOSINE	Ki (nM)	
NBMPR	3.4 (2.2 - 6.9)	
Dipyridamole	70 (58 - 94)	

Inhibition constants are the geometric means (95 % confidence limits) of Ki values obtained obtained from 3 separate experiments performed in quadruplicate.

IV. DISCUSSION

Although many studies have measured nucleoside "uptake" in heart, the present study is the first to examine the transport of nucleosides into cardiac cells. In studies that fail to measure initial rates of nucleoside uptake, the measurements of permeant influx can be influenced by permeant metabolism, counterfluxes of permeant or metabolites, or diffusion of the permeant through the extracellular compartments of the tissue to the site of transport. The use of a dissociated ventricular myocyte preparation for the present study allowed (a) direct measurements of nucleoside transport using "initial uptake rate" methodology, and (b) indirect study using NBMPR binding techniques.

A comparison of drug-induced inhibition of NBMPR binding and nucleoside transport validated the use of NBMPR as a high affinity binding probe to study the nucleoside transport system in heart. The affinity of a number of drugs for NBMPR sites was found to be predictive of their abilities to inhibit nucleoside transport, indicating that NBMPR binding sites are inhibitory sites associated with the nucleoside transport system.

Species differences in both nucleoside transport and NBMPR binding were found. Rat myocytes, in comparison to guinea pig myocytes, had a significantly lower maximal NBMPR binding capacity and also possessed a lower maximal rate of nucleoside transport. In rat myocytes, dipyridamole

possessed a lower affinity for both NBMPR sites and the transport process as compared to guinea pig myocytes. Conversely, adenosine possessed a higher affinity for NBMPR sites and the transport process in rat myocytes than in guinea pig myocytes. The present study indicates that in heart tissue heterogeneous transporters exist. Further, 2-chloroadenosine was found to be a substrate for the nucleoside transport process.

A. Tissue Preparations and Methods

Ventricular Myocyte Preparation

The method chosen for the preparation of both guinea pig and rat myocytes was a collagenase digestion procedure using the Langendorf perfusion technique, followed by mechanical aggitation. Unfortunately, there are many unknown factors involved in the preparation of myocytes. For example, it has been shown that contaminants in crude collagenase, contribute to the disruption of connective tissues during myocyte preparation (Kono 1969; Dow *et al.* 1981). Collagenase concentrations, media and incubation times were chosen for this study solely to produce the highest possible quality of cells. The final method produced relatively high cell yields (approximately 10 million cells per heart) and between 70 and 85% viability (as determined by the rod-shaped appearance and trypan blue exclusion of viable cells). Within experiments that

employed myocytes in replicate assay mixtures, the order in which those mixtures were assembled was randomized in order to avoid a bias due to the time-dependent loss of viability (< 5% per hour) in the myocyte preparations.

A possible disadvantage of the procedure chosen for myocyte preparation is the lack of tolerance of the cells obtained to physiological calcium ion concentrations. This phenomenon is known as the calcium paradox. This occurs when cells are exposed to calcium-free medium and, following the reintroduction of calcium, substantial damage to cell membranes occurs (Dow *et al.* 1981). It is important to note, however, that the absence of calcium does not seem to influence [^{3}H]NBMPR binding (Hammond 1983) or nucleoside transport (Ford *et al.* 1985). High yields of calcium-tolerant ventricular myocytes would have been difficult to obtain. Many of the studies reporting the preparation of calcium-tolerant myocytes (a) often do not require high percent cell viability (as in the use of myocytes for electrophysiological studies) or (b) produce viable cells in too few a number to be useful in biochemical assays. In light of more recent reports of calcium-tolerant preparations (Lundgren *et al.* 1984), it may be advantageous to pursue such methods in the future.

Collagenase Treatment

Collagenase dissociation procedures are commonly used in the preparation of cells for electrophysiological

(Belardinelli and Isenberg 1983) and biochemical (Capps *et al*. 1985; Wong and Ooi 1985) measurements. It has been reported that cells prepared with crude protease preparations may lose their glycocalix layer and often their surface proteins (Ryan *et al*. 1980). Therefore, it is conceivable that the use of crude collagenase preparations to dissociate myocytes from the connective tissue and other cells of the heart may degrade cell surface proteins associated with NBMPR binding sites and/or the nucleoside transport system. Lowered NBMPR site density in collagenase-treated guinea pig membranes and lowered NBMPR affinity in collagenase-treated rat membranes, as compared to paired control preparations, would indicate that collagenase treatment may have affected NBMPR binding proteins. Although it is conceivable that proteolytic damage during collagenase treatment could have reduced the affinity of dipyridamole for the nucleoside transport system in rat myocytes, this is unlikely for the following reasons:

(1) The conditions to which the collagenase treated membranes were exposed in this study were probably much harsher than the myocyte preparation conditions;

(2) while ventricular myocyte preparations contain only one type of cell, ventricular membrane preparations contain fragments of a variety of cells including smooth muscle and endothelium. It is possible that collagenase treatment may not

have a similar effect on all cellular components; (3) a previous report of experiments with cardiac membrane preparations (Williams *et al*. 1984) in the absence of collagenase treatment has indicated that (a) species differences exist with respect to dipyridamole affinity for NBMPR sites, (a lower affinity for sites in rat membranes as compared to those in guinea pig membranes was observed), and (b) species differences exist with respect to adenosine affinity for NBMPR sites. The higher affinity of adenosine for NBMPR sites on rat myocytes as compared to guinea pig myocytes is also consistent with previous findings in heart (Williams *et al*. 1984).

In addition, as both NBMPR binding and adenosine transport can be examined using ventricular myocytes, comparisons of these processes, irrespective of the effects of collagenase, may still be made, and thus, provide useful knowledge of the functional characteristics of NBMPR sites and nucleoside transport in cardiac cells.

NBMPR Binding Techniques

Although ligand binding experiments are more commonly performed using crude membrane preparations, the study of NBMPR binding to intact cardiac cells did not present any apparent difficulties. An additional benefit of using myocytes was that bound ligand could be rapidly removed from

free ligand by rapid (15s) centrifugation. Since the use of viable dissociated myocytes was mandatory for the measurement of nucleoside influx rates, this tissue was also used to study NBMPR binding so that these processes could be directly compared in the same preparation.

Free [^{3}H]NBMPR was calculated by subtracting total binding (after one wash of the pellet) from the initial amount of ligand added to the assay. This value may be an overestimation of the actual free ligand concentration at equilibrium, since the washing step may remove nonspecific binding, and thus, decrease total binding. A better method for determining free ligand concentration at equilibrium would be to assay the supernatant concentration of ligand following centrifugation, but prior to washing the pellet.

Nucleoside Influx Studies

Time courses of nucleoside uptake represent the accumulation of radiolabelled substrate over relatively long (several min) incubation intervals. These measurements are often influenced by other processes besides the facilitated transport of nucleosides, as has been described in the introduction section of this thesis. For this reason, radioactivity derived from radiolabelled nucleosides often concentrates inside cells during long incubation intervals. Time-course experiments were used to determine the time intervals which permitted the measurement of initial rates of nucleoside influx. A 15s incubation interval, chosen for

the transport studies, ensured that initial velocities of influx for each substrate concentration were measured and that nucleoside uptake was less than 20% of the value at which the intra- and extracellular substrate concentrations would have been at equilibrium.

The "cold-inhibitor-stop" used to terminate nucleoside influx intervals was effective and immediate, as indicated by the fact that time-courses of nucleoside uptake extrapolated through time zero. High concentrations of nucleoside transport inhibitors have been shown to stop the influx of nucleosides rapidly (Paterson *et al.* 1983). In the present experiments, in addition to NBMPR and dilazep, a high concentration of unlabelled permeant was used in the stopping mixture. This reduced the specific radioactivity of the extracellular labelled permeant and thus, minimized inward diffusion of radiolabel.

The component of nucleoside influx that was not inhibited by high concentrations of nucleoside transport inhibitor (such as 20 μM NBMPR or 100 μM dilazep) is most likely due to the passive diffusion of the nucleoside. Therefore, transporter-mediated entry was determined as the difference in the uptake of radiolabel in the absence (total uptake) or presence (nonmediated uptake) of nucleoside transport inhibitor.

B. NBMPR Binding Constants in Cardiac Membranes and Myocytes

The binding constants obtained in the present study indicate that NBMPR binds with high affinity to an apparent single class of sites on both ventricular membranes and ventricular myocytes from guinea pig and rat. The product of adenosine deamination, Inosine, has been shown to possess a lower (3- to 4-fold) affinity for NBMPR sites than adenosine in guinea pig brain (Hammond 1983). NBMPR binding was unaffected by adenosine deaminase, indicating that adenosine was not being released from cells in the assays in concentrations sufficient to compete with NBMPR for its binding sites. The Kd and Bmax values for [^{3}H]NBMPR binding to guinea pig and rat ventricular membranes were similar to those of Williams *et al.* (1984). However, these species exhibit differences in maximum binding capacity in both membrane and myocyte preparations. While an 8-fold greater maximum [^{3}H]NBMPR binding capacity (Bmax) was obtained in guinea pig membranes, as compared to rat membranes, only a 2-fold difference was observed with myocyte preparations from these species. This may be explained by the differences in composition of these two preparations. Cardiac membranes (P_2 fraction) are a crude preparation composed of fragments of many cell types; while the ventricular myocyte preparation contains cells of only one type. It is important that this distinction be made when comparing results obtained with crude heart membranes or intact heart and those obtained with ventricular myocytes.

C. Nucleoside Transporter and NBMPR Binding Sites

Adenosine and 2-chloroadenosine are substrates for the nucleoside transport system in myocytes and enter cells via a saturable and inhibitable process. These nucleosides bind in an apparent competitive manner to NBMPR sites on these cells and on membranes, indicating indirectly that they have affinity for the transporter in cardiac tissue. Definitive evidence that both adenosine and 2-chloroadenosine are substrates for the cardiac nucleoside transporter was obtained by measuring nucleoside fluxes directly in myocytes. The result showing that NBMPR or dipyridamole possessed a similar potency to inhibit adenosine and 2-chloroadenosine transport into guinea pig myocytes is evidence that both nucleosides are substrates for the same transport process. A recent study has shown that 2-chloroadenosine is a substrate for the nucleoside transport system in human erythrocytes (Jarvis *et al*. 1985). It was shown that the Km for 2-chloroadenosine transport was similar to the Ki for 2-chloroadenosine-mediated inhibition of uridine transport (Jarvis *et al*. 1985). This is further evidence that 2-chloroadenosine is a substrate for the facilitated diffusion nucleoside transport system in mammalian tissue. The demonstration that 2-chloroadenosine binds to NBMPR sites (Ki= 14μM) on guinea pig CNS membranes (Hammond and Clanachan 1984a) would suggest that it may also be a substrate for the nucleoside transport process in brain. It is clear that the failure of nucleoside transport

inhibitors to potentiate adenosine receptor-mediated effects of 2-chloroadenosine may no longer be attributed to 2-chloroadenosine's inability to be carried by the nucleoside transporter. This will be discussed subsequently.

2-Chloroadenosine possesses a higher affinity than adenosine for the transport process in guinea pig myocytes and its Km (36μM) for transport is similar to its Ki (22μM) for inhibition of [^{3}H]NBMPR binding. These values are in close agreement with those obtained by Jarvis and associates (1985) with human erythrocytes. The affinities of adenosine for the transport processes in guinea pig (Km = 146μM) and rat (Km = 50μM) myocytes are in a similar range to values reported in recent studies of adenosine transport in a variety of cell types (Lum *et al*. 1979), but also of lower affinity than values reported in other studies (Paterson *et al*. 1985). A number of other studies using heart tissue (Hopkins and Goldie 1971; Olsson *et al*. 1972; Mustafa 1979; Bowditch *et al*. 1985a) have reported higher affinities (Km values of 1 to 10μM) for adenosine. However, these studies employed long incubation intervals and thus were most likely measuring the total intracellular accumulation of substrate and metabolites rather than initial rates of adenosine influx. The Km values reported for adenosine accumulation in those studies are in close agreement with the Km of adenosine for adenosine kinase (0.5 to 5μM).

Adenosine kinase was most likely responsible for the apparent concentration of radiolabelled permeant seen at longer incubation intervals (greater than 60s) used during time-course protocols in the present experiments with myocytes. This may have been due to the intracellular conversion of adenosine to impermeable nucleotides. The role of adenosine kinase is confirmed by the demonstration that in guinea pig myocytes the radiolabel derived from low concentrations of adenosine (such as 1μM) close to the Km for adenosine kinase concentrated to a greater degree than higher concentrations (100μM) which would have saturated the enzyme. It is unclear whether 2-chloroadenosine is subject to phosphorylation in guinea pig cardiac myocytes. We failed to show any concentrating effect with 100μM 2-chloroadenosine, while 1μM 2-chloroadenosine concentrated slightly (1.5-fold). However, it is unclear whether the latter was due to kinase activity or error in estimating intra- and extracellular substrate concentrations. Other workers (Yamanaka *et al.* 1984) have shown that it is a substrate for adenosine kinase (and other kinases) in cultured human splenic B lymphoid cells. It is clear, however, that initial rate methodology is necessary to make direct measurements of nucleoside transport without interference of other metabolic processes.

The maximum velocity of adenosine transport is a function of the carrying capacity of each individual transporter as well as the total number of transport sites.

The current finding that guinea pig myocytes possess a 2-fold greater maximum [^{3}H]NBMPR binding capacity and a 3-fold greater maximum velocity of [^{3}H]adenosine transport, as compared to rat myocytes, indicates that [^{3}H]NBMPR binding site density should be predictive of the density of transport sites. This is evidence that, in these cells, NBMPR sites are closely associated with the nucleoside transport system, as has been previously demonstrated for some other cells, such as erythrocytes. Preliminary evidence that NBMPR sites are closely associated with functional nucleoside transporters in heart has previously been reported (Williams *et al.* 1984). The finding that guinea pig membranes possessed an 8-fold greater [^{3}H]NBMPR binding capacity, as compared to rat membranes, was consistent with the 6-fold difference in "nucleoside transport-dependent accumulation" of [^{3}H]adenosine into intact ventricular "chunks" from these species.

The adenosine translocation capacities for guinea pig and rat myocytes, calculated by dividing the maximum velocity of nucleoside transport by the maximum NBMPR site density (assuming a one-to-one relationship between NBMPR sites and functional transporters), are approximately 17 molecules of adenosine per site per s and 10 molecules of adenosine per site per s, respectively. The values obtained with myocytes are lower than those obtained for the translocation capacity of adenosine in human erythrocytes (approximately 60 molecules adenosine per site per s; Jarvis

et al. 1982a; Jarvis *et al.* 1985). The 2-chloroadenosine translocation capacity in guinea pig ventricular myocytes is approximately 8 molecules per site per s; which is also less than that obtained (Jarvis *et al.* 1985) for human erythrocytes (33 molecules per site per s). Thus it can be concluded that cardiac myocytes, like many species of erythrocytes (such as human), possess high affinity NBMPR sites that are closely and functionally associated with the nucleoside transport system. However, the translocation capacity of cardiac transporters is lower than those in erythrocytes.

D. Drug Affinites for NBMPR Binding Sites and the Nucleoside Transport Process

Species differences exist with respect to the affinity of various known nucleoside transport inhibitors and nucleosides for NBMPR sites in heart. While diazepam bound with similar affinity to NBMPR sites in both guinea pig and rat myocytes, dipyridamole, and to a lesser extent, dilazep possessed a higher affinity for NBMPR sites in guinea pig, as compared to rat. This indicates that heterogeneous NBMPR sites may exist in cardiac cells. Dipyridamole has also been shown to possess a lower affinity for NBMPR sites in cardiac membanes (Williams *et al.* 1984), brain (Hammond and Clanachan 1985) and lung (Shi *et al.* 1984) from rat, as compared to guinea pig. Previous studies have shown that dipyridamole is also a much weaker inhibitor of adenosine

"uptake" into rat erythrocytes (Kolassa and Pfleger 1975) and rat ventricular "chunks" (Williams *et al*. 1984) than into similar preparations from guinea pig.

Drug affinity for NBMPR binding sites on ventricular myocytes has been found to be predictive of the agents ability to inhibit nucleoside transport in these cells. In guinea pig myocytes, the Ki values for dipyridamole-, dilazep- and diazepam-mediated inhibition of [^{3}H]NBMPR binding and [^{3}H]adenosine transport are similar. In rat myocytes, this was also the case for dipyridamole and diazepam. However, in rat, dilazep possessed a greater potency to inhibit NBMPR binding than to inhibit adenosine transport. A possible explanation for this may be that dilazep was partially metabolized by esterases released from cells in the transport assays (which possessed a much larger cell density than NBMPR binding assays). However, it is unknown why rat esterase activity would differ from that in guinea pig myocytes. Dipyridamole is not a substrate for esterases and did not show differing potencies for inhibition of NBMPR binding and adenosine transport in either species. The results indicate that good correllations exist between the drug affinities determined from NBMPR binding and nucleoside transport methodologies (Table 6). This would indicate that, in myocytes, NBMPR binding sites are inhibitory sites associated with the nucleoside transport system and that NBMPR site occupancy results in inhibition of nucleoside transport. Preliminary

Table 6. Comparison of the affinities of NBMPR, dipyridamole, dilazep, diazepam, adenosine and 2-chloroadenosine for the nucleoside transport systems of guinea pig and rat myocytes derived from [^{3}H]NBMPR binding studies and from transport studies utilizing [^{3}H]substrates.

Drug	Guinea pig		Rat	
	Binding	Transport	Binding	Transport
NBMPR	0.76 nM[1]	2.8 nM	0.40 nM[1]	2.4 nM
Dipyridamole	75 nM	78 nM	1700 nM	3600 nM
Dilazep	6.8 nM	8.8 nM	34 nM	260 nM
Diazepam	36 μM	27 μM	43 μM	52 μM
Adenosine	318 μM	146 μM[2]	35 μM	50 μM[2]
2-Chloroadenosine	22 μM	36 μM[2]		

Values are geometric means of 3 to 6 separate experiments in duplicate. Drug affinities for the nucleoside transporter are represented by Kd[1], Km[2] or Ki values as appropriate.

evidence for this observation has previously been presented for heart by Williams and associates (1984), who found that the affinities of various transport inhibitors for NBMPR sites on guinea pig cardiac tissue were similar to those obtained for inhibition of "nucleoside transport-dependent accumulation" of adenosine. The results obtained with ventricular myocytes argue that NBMPR sites in heart tissue are functionally coupled to the nucleoside transport system in an inhibitory manner.

Inhibitor depletion was not accounted for in transport inhibition assays. Depletion due to specific binding to NBMPR sites in assays containing 500,000 cells can be calculated to be as much as 0.7 pmoles and 0.4 pmoles with guinea pig and rat myocytes, respectively. Nonspecific binding of inhibitors may result in additional depletion in these assays. Taking into account this loss of free inhibitor, the inhibition constants for NBMPR-mediated inhibition of nucleoside transport would be much closer to the Kd values for NBMPR binding.

Species differences exist with respect to the affinity of adenosine for the nucleoside transport system. Adenosine possessed a higher affinity for NBMPR sites on rat myocytes, as compared to guinea pig myocytes, and also possessed a higher affinity for the nucleoside transport process in rat than guinea pig. Adenosine-mediated inhibition of NBMPR binding was not affected by deoxycoformycin (an adenosine deaminase inhibitor) in either species of myocytes, and

therefore, it is unlikely that adenosine was being metabolised by endogenous adenosine deaminase in these assays. In addition, intracellular metabolism of the permeant was avoided in transport assays by using "initial rate" methodology (Paterson *et al*, 1981). The similar Ki and Km values obtained for adenosine in both species would indicate that the inhibitory site and the permeation site of the nucleoside transport complex are similar. This is further substantiated by the finding that 2-chloroadenosine also possessed a similar affinity for NBMPR sites as for the transport process.

E. Potentiation of Nucleoside Actions

The demonstration that 2-chloroadenosine and adenosine both enter heart cells by a saturable process, which is inhibited by agents such as NBMPR or dipyridamole, whereas only the actions of adenosine are potentiated by nucleoside transport inhibitors, would appear to raise doubts concerning the mechanism of transport inhibitor-induced potentiation of adenosine actions. However, using the transport kinetic constants obtained for 2-chloroadenosine and adenosine and their IC_{50} concentrations (Clanachan and Marshall 1980) for the reduction of isometric tension of electrically driven guinea pig left atria (0.05μM and 191μM, respectively), the velocities of inhibitable transport of these substrates can be calculated to be 0.016 pmoles 2-chloroadenosine/10^6 cells/s and 13.6 pmoles adenosine/10^6

cells/s. This indicates that at their equi-effective concentrations in heart tissue, the velocity of adenosine transport would be approximately 850-fold greater than that of 2-chloroadenosine. This difference may be further augmented at prolonged incubation intervals (greater than 60s) used in contractility studies, where total inhibitable entry of adenosine may be additionally enhanced by concentration effects due to intracellular metabolic conversion to impermeable nucleotides. Therefore, in experimental systems where 2-chloroadenosine possesses a higher potency than adenosine (such as in heart), the removal of nucleoside from the vicinity of adenosine receptors by transport and subsequent intracellular metabolism would be lower for 2-chloroadenosine than for adenosine. Therefore, it should not be unexpected that nucleoside transport inhibitors exert a greater potentiation of the effects of adenosine than those of 2-chloroadenosine. Interestingly, calculation of the transport velocities of these two substrates at comparable concentrations less than 70μM reveals that the influx velocity of 2-chloroadenosine is slightly greater than that of adenosine. Therefore, in systems where adenosine and 2-chloroadenosine possess equivalent potencies at adenosine receptors, it should be expected that both agents would be potentiated by nucleoside transport inhibitors in a similar manner.

F. Conclusions

Dissociated ventricular myocytes from guinea pig and rat were used to examine [^{3}H]NBMPR binding and initial rates of nucleoside uptake. [^{3}H]NBMPR bound with high affinity to a single class of sites in both species. Adenosine and 2-chloroadenosine were found to be substrates for the cardiac nucleoside transport process, entering cells in a saturable and inhibitable manner. Following long incubation intervals, the radioactivity derived from radiolabelled adenosine concentrated within myocytes, indicating that it may have been phosphorylated. For guinea pig and rat myocytes, the maximum NBMPR binding site densities (Bmax) were about proportional to the maximum adenosine transport capacity (Vmax), suggesting that NBMPR sites are closely associated with the nucleoside transport system. The similar drug potencies for inhibition of NBMPR binding and inhibition of nucleoside transport demonstrate that NBMPR sites are transport inhibitory sites in ventricular myocytes, i.e. NBMPR binding site occupancy results in inhibition of nucleoside transport in these cells. In addition, the similar affinities of substrates (adenosine and 2-chloroadenosine) for NBMPR sites and the transport process indicate that NBMPR sites and the transport permeation site may be similar (or closely associated). This study demonstrates that NBMPR is a useful high affinity binding probe for the nucleoside transport system in ventricular myocytes.

Initial rate measurements of 2-chloroadenosine uptake confirmed that it is substrate for the transporter in guinea pig myocytes. This was predicted by the finding that 2-chloroadenosine possessed affinity for NBMPR sites in ventricular myocytes. The lack of potentiation of 2-chloroadenosine actions at adenosine receptors by nucleoside transport inhibitors may be explained by its low transport velocity at concentrations which would cause receptor stimulation.

This study has not examined symmetry of the nucleoside transporter activity with respect to substrates or inhibitors. However, ventricular myocytes may be a good preparation to examine these characteristics. With this preparation, it may be possible to study inhibitor-induced modification of the release and actions of endogenous adenosine (such as during hypoxia-induced hyperemia).

This study has demonstrated the existence of species differences with respect to nucleoside transport site density and transport capacity (Bmax and Vmax) between guinea pig and rat myocytes. In addition, significant differences in drug affinity for the transport system were obtained between these species. Should heterogeneity of transport exist within a species or within an organ, it may be possible to modify selectively the actions of adenosine. If differences in transporter site density exist, transport inhibitors may be utilized to produce greater potentiation in areas where site density is greater and therefore

transport capacity is also greater. The discovery of differences in drug affinity for transporters within a species or organ, as determined by the use of binding probes such as NBMPR, may lead to the development of selective potentiators of adenosine action through selective inhibition of adenosine transport.

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APPENDIX 1

A. Composition of Buffers and Liquid Scintillation Fluors.

(1) Composition of Assay Medium (Modified Dulbecco's PBS)

NaCl	(137 mM)	8 g/l
$Na_2HPO_4.7H_2O$	(6.3 mM)	1.7 g/l
KCl	(2.7 mM)	200 mg/l
KH_2PO_4	(1.5 mM)	200 mg/l
$MgCl_2.6H_2O$	(0.5 mM)	100 mg/l
Glucose	(10 mM)	1.8 g/l
PVP-40	(5 %)	50 g/l

Adjust to pH 7.4 with NaOH. PVP-40 = polyvinylpyrolidone. (from Dulbecco & Vogt 1954)

(2) Composition of Joklik Medium

$MgSO_4.7H_2O$	(1.2 mM)	0.296 g/l
D-L Carnitine	(1.0 mM)	0.198 g/l
Na Bicarbonate	(23.8 mM)	2.0 g/l
Adenosine	(0.1 mM)	27 mg/l
Taurine	(60 mM)	7.5 g/l
Glucose	(50 mM)	9.0 g/l

Include 1 package of MEM Culture Medium (Joklik-Modified), from Gibco Laboratories, in 1 litre of medium. Carbogenate and adjust pH to 7.4 with NaOH.

(3) Composition of Digestion Medium

To 50 ml of Joklik medium add:

a) 300 u/ml Sigma type V collagenase

b) 150 u/ml Sigma type IA collagenase

c) 50 mg (0.1 %) Bovine Serum Albumin (BSA)

(4) Composition of KB Medium

KCl	(85 mM)	6.4 g/l
K_2HPO_4	(30 mM)	5.25 g/l
$MgSO_4.7H_2O$	(5 mM)	1.2g/l
Taurine	(60 mM)	7.5 g/l
Glucose	(50 mM)	9.0 g/l

Adjust pH to 7.4 with KOH.

(5) Disruption Medium

To 98 ml KB medium add:

PVP-40	(5 %)	5.0 g
ATP	(5 mM)	0.25 g
Pyruvate	(5 mM)	55 mg
EGTA	(40 μM)	2 ml of 2 mM stock

(6) Tritisol Scintillant (4 litres)

PPO	12 g
POPOP	800 mg
Xylenes	2300 ml
Triton X-100	1000 ml
Ethanol (98 %)	560 ml
Ethylene glycol	140 ml

PPO = 2,5-Diphenyloxazole
POPOP = 1,4-Bis(5-Phenyloxazol-2-yl)-Benzene

(7) Toluene Scintillant (2 litres)

PPO	10 g
POPOP	400 mg
Ethylene glycol MME	600 ml
Toluene	1200 ml

MME = monomethyl ether